THE UNIVERSITY
of EDINBURGH

RNA interference in parasitic nematodes – from genome to control

Thomas Tzelos

Thesis submitted for the degree of Doctor of Philosophy to

The College of Medicine and Veterinary Medicine

The University of Edinburgh

Research was carried out at Moredun Research Institute

Doctor of Philosophy – The University of Edinburgh – 2014

Contents

List of Figures

List of Tables

Declaration

The work presented in this thesis is my own work, unless otherwise stated, and has not been submitted for any other degree.

Thomas Tzelos

Acknowledgements

Firstly, I would like to express my most sincere gratitude to my supervisors, Professors Dave Knox, Jacqui Matthews and Bruce Whitelaw, for their invaluable advice, support, guidance and constant encouragement over the past years. I would also like to thank Zoetis and Easter Bush Research Consortium (EBRC) for providing the funding for the research.

I am very grateful to several people at Moredun Research Institute - Dave Bartley, Tom McNeilly, Alison Morrison, Leigh Devin, Yvonne Bartley, Margaret Oliver, Chris Cousens, Neil Inglis and David Frew - for their help and support in my project. A special thank you to Dr Al Nisbet for his priceless the support throughout my project. Thanks are also due to the other PhD students; Claire, Ru, Cassie, Frank, Heather and Valentina, for their encouragement to help me get through the most stressful days. Moreover, I would like to thank Dr Amy Buck's lab (King's Buildings, The University of Edinburgh) and especially Dr Fabio Simbari and Gillian Coakley for their help in the final part of the project. A big thank you is due to my beloved Dr Eleni Maria Amaniti who stood by me and for all her support.

Finally, a huge thank you to my parents (Nikos and Dimitra), my brother (George), and my sister (Evi) for always supporting my decisions on my personal and professional life.

Abstract

Teladorsagia circumcincta is a parasitic nematode which is a major cause of ovine parasitic gastroenteritis in temperate climatic regions. The parasite has developed resistance to the major anthelmintic drug classes and this challenges its future control. Vaccination is a potential alternative control method since sheep are able to develop protective immunity against this parasite. Although potential vaccine candidates have been revealed, the increasing gene datasets suggest that vaccine-target selection may be aided by screening methods such as RNAi. This is a reverse genetic mechanism that causes highly specific gene silencing which was initially described and applied to defining gene function in *Caenorhabditis elegans*. Nevertheless, its application was more difficult than anticipated in parasitic nematodes because of the inconsistency of the silencing effect. In the unsuccessful cases, did the dsRNA penetrate the parasite and activate the RNAi pathway? Thus far, there are no internal controls that indicate the activation of the pathway. Are the RNAi pathway genes constantly transcribed or are they 'switched on' in response to the dsRNA exposure? The initial aim of the study was to determine potential marker genes in the RNAi pathway that could indicate the activation of the pathway in *C. elegans*. After the exposure to dsRNA from two target genes, the transcript levels of three candidate marker genes (*Ce-dcr-1*, *Ce-ego-1* and *Ce-rsd-3*) were examined and showed that exposure to dsRNA has no effect on the transcript levels of these genes making them inappropriate markers for the activation of the RNAi pathway. The two target-genes were *Ce-cpr-4* and *Ce-sod-4* which had been proven to be consistently susceptible and refractory to RNAi, respectively. Another aim of the project was to develop an RNAi platform in *T. circumcincta* for use as a screening method for potential vaccine candidates. The targets selected for the *in vitro* RNAi included: five members of the Activation-associated Secreted Proteins (ASPs); a Macrophage migration Inhibitory Factor-like (*Tci-mif-1*) and a Surface Associated Antigen gene (*Tci-saa-1*), all of which have been associated with vaccine-induced protective immunity. The selection of the ASPs was based on a bioinformatic and transcriptomic analysis of the ASPs in *T. circumcincta*. The results showed successful knock-down only for three out of five ASP targets after 1 hour of soaking

in gene-specific double stranded RNA (dsRNA) which illustrates the inconsistency and the target specificity of RNAi in *T. circumcincta* which has been observed in the past with other parasitic nematodes. Inconsistencies were also observed within the successful ASP targets with the results not being reproducible after several successful experiments. Potential reasons for the inconsistencies were examined with the duration of larval storage being a critical factor. Larvae stored for a short or long period of time were susceptible and refractory to RNAi, respectively. Experiments were also conducted to investigate how the ASPs relate to extracellular microvesicles (EMVs). These vesicles are considered to play an important role in the intercellular communication between parasites and their hosts, and thus represent potentially useful vaccine and/or drug targets. Transmission electron microscopy (TEM) confirmed that EMVs are excreted / secreted by the parasite and the proteomic analysis revealed several types of proteins within the vesicles such as: ASPs, Actins, Metallopeptidases, and RAB proteins. A comparative analysis of EMVs, EMV-free ES (Excretory / Secretory) and total ES products showed that approximately 35% of the proteins found in the vesicles could also be identified in EMV-free ES and in total ES products, whilst the remaining 65% were present only in EMVs.

Chapter 1: Review of the literature

1.1 Parasites

Parasitism is a type of symbiosis which involves two organisms, the parasite and the host. In this type of relationship the parasite benefits at the expense of the host. Parasites can be classified as ecto- or endo-parasites depending on whether they live on the surface or inside their host, respectively. Endoparasites include the protozoa, which are single cell organisms and generally live in host cells, and metazoa, which are multicellular organisms living in systems within the host. Metazoan parasites are further divided to Nemathelminthes (nematodes or roundworms) and Platyhelminthes, which include the Cestodes (tapeworms) and Trematodes (flukes).

1.2 The parasitic nematode *Teladorsagia (*formerly *Ostertagia) circumcincta*

1.2.1 Taxonomical classification and life cycle

Teladorsagia (formerly *Ostertagia*) *circumcincta* (Stadelmann, 1894) is a parasitic nematode with a taxonomic status as shown in Figure 1. 1.

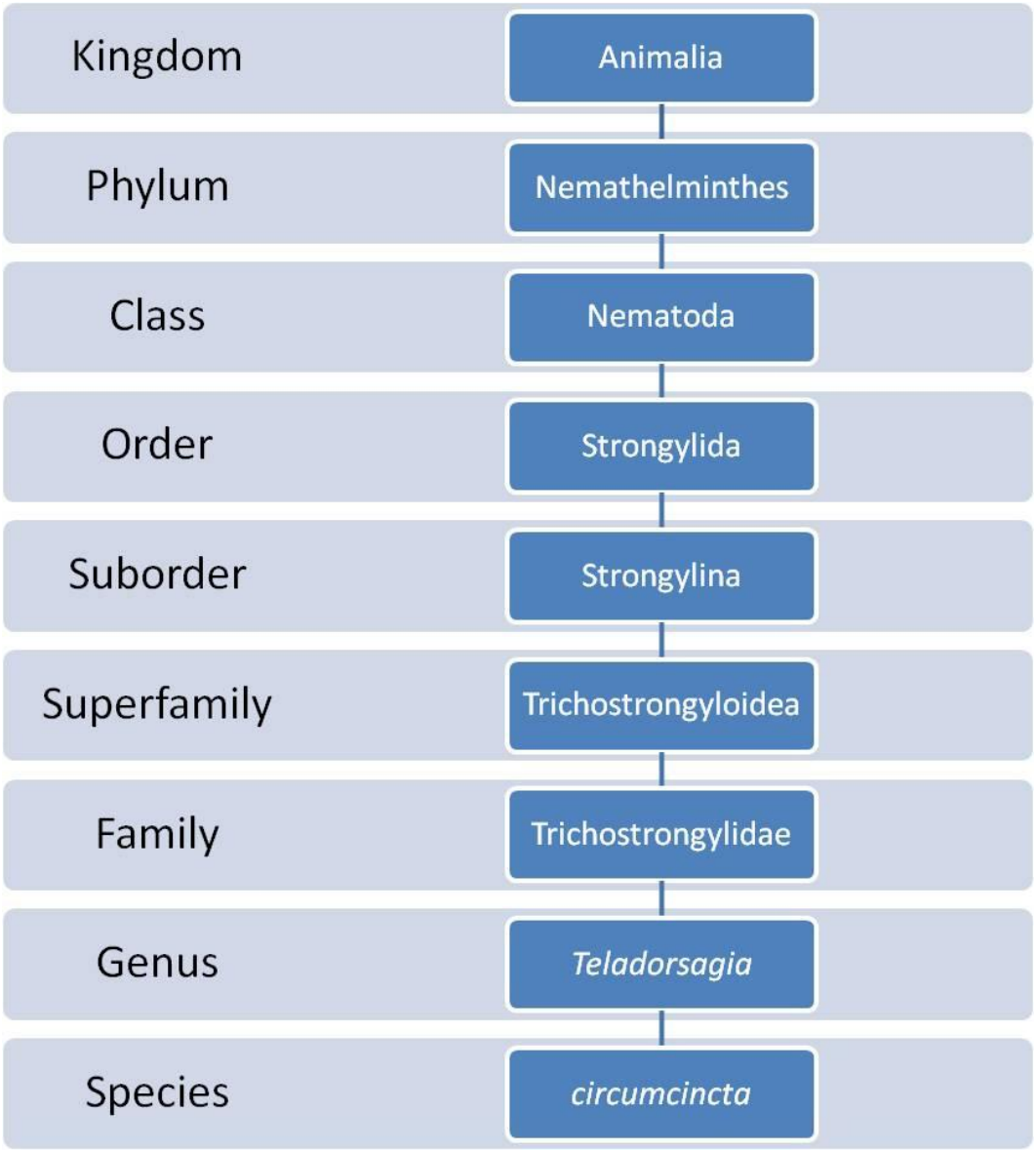


Figure 1.1. Taxonomic status of *Teladorsagia circumcincta*

T. circumcincta inhabits the abomasum of small ruminants (sheep and goats). The development of this parasite was initially examined by Threlkeld (1934) and its morphology and life cycle has been described in many parasitology books (Threlkeld, 1934, Anderson, 2000, Taylor *et al.*, 2007). The parasite has a direct life cycle (Figure 1. 2.) which lasts approximately five weeks. Initially, the eggs are passed to the environment in the faeces. The eggs hatch and the first stage larvae (L1) emerge. These undergo two moults and develop to infective third stage larvae (sheathed L3) in the faecal pat. Hatching and moulting are temperature and humidity

dependent and eggs can develop to L3 in as short as 2 weeks under optimum conditions (Crofton and Whitlock, 1965). L3 migrate from the faeces to the herbage and are ingested by the host. In the host, they moult as they pass through the rumen into the abomasum where they invade the abomasal glands in the mucosa. They cause the production of nodules and swellings containing one or more larvae (Moroccan leather nodules) where they develop through a fourth larval stage (L4) to the pre-adult (L5) stage (Sommerville, 1953). The parasites emerge onto the abomasal surface where they become sexually mature adults and another life cycle begins after copulation. Development of the larvae within the host has been described in detail by Denham (1969): larvae moult to L4 stage in the abomasal glands four days post infection (dpi). Throughout the first four dpi, the growth of the parasites is relatively slow. Their length increases by less than 0.5 mm and by the 4^{th} dpi, the average length is 1.07 mm. The worms remain at this stage for four further days and then moult to the L5 stage around the 8^{th} dpi. The first immature adult females that start carrying eggs can be observed on the 12^{th} dpi. The growth of the parasites after the 4^{th} dpi is rapid. The length of the female and the male parasites by the 14^{th} dpi is 12 to 13 mm and 8 to 10 mm, respectively. Finally, the L5 parasites emerge onto the abomasal mucosal surface around the 18^{th} dpi where they develop further to sexually mature adults and start laying eggs (Denham, 1969). Usually development from L3 to adult worms within the host takes approximately 3 weeks. However, if the infective L3 larvae are ingested in mid-Autumn, they may inhibit their development when they reach the early L4 stage and this may persist for periods up to six months. The subject of inhibited development in parasitic nematodes has been reviewed by many (Michel, 1978, Gibbs, 1986). Inhibited development allows the parasites to survive inside the host during the adverse winter months. Although the exact stimuli that lead *T. circumcincta* to enter or exit the inhibited state remain unknown, studies have shown that some of the potential stimuli include the climatological changes (photoperiod) and the immunological status of the animal due to parturition and lactation (Smith, 2007, Langrová *et al.*, 2008).

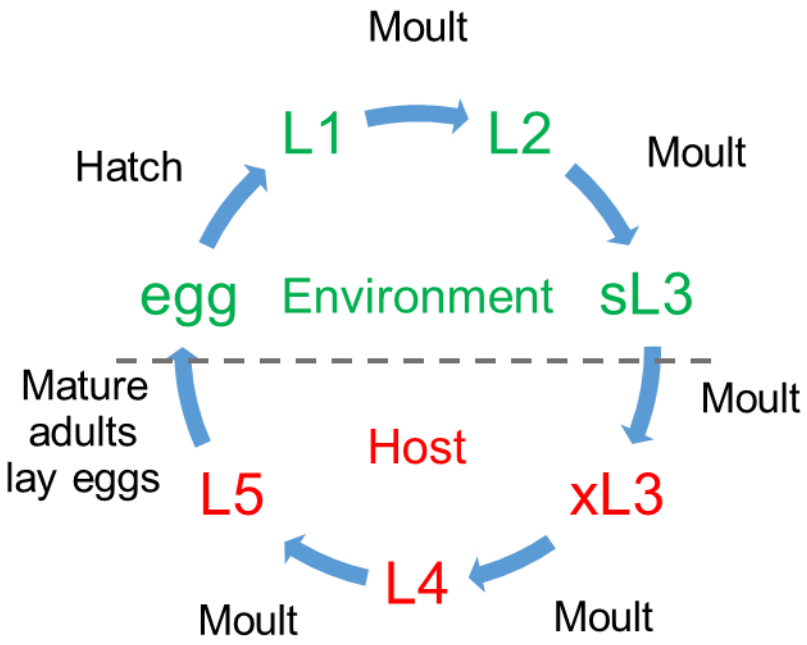


Figure 1.2. Life cycle of *T. circumcincta*

1.2.2 Clinical implications and pathophysiology

T. circumcincta is the principal cause of ovine parasitic gastroenteritis (teladorsagiosis) in temperate climatic regions of the world and has been reported as the most predominant nematode in small ruminant flocks in the UK (Bartley *et al.*, 2003, Burgess *et al.*, 2012). In the northern hemisphere the disease is usually seen from July to October, a time period which coincides with the exponential increase of the infective L3 *T. circumcincta* in the environment. The principal targets of the parasite are growing young stock. It was suggested that adult sheep are more resistant to nematode infection, and also that breeding ewes contaminate the pasture with significant numbers of eggs during the periparturient period when a relaxation in the immune responses is observed (Donaldson *et al.*, 1998, Houdijk *et al.*, 2001, Huntley *et al.*, 2004). A recent study has developed a model which has shown that the pasture is mainly contaminated with eggs that develop to infective larvae derived from the ewes (Singleton *et al.*, 2011). The lambs contribute to the contamination of the pasture when they are 2-3 months old due to the nematode development time and

the low ingestion rates (Singleton *et al.*, 2011). The parasite numbers increase with the further development of the larvae to adult parasites in the lambs and the subsequent production of eggs (Stear *et al.*, 2007).

The main clinical manifestations of parasitic gastroenteritis are reduced weight gain/condition loss and the dehydration of the animals due to profuse watery diarrhoea which finally leads to emaciation of the animals (Scott, 2007). Except from the gastrointestinal problems, the faecal contamination of the fleece in the perineal area may attract blowflies, which can lead to myiasis. Teladorsagiosis not only jeopardises the welfare of the animals, but also has a significant economic impact on the sheep farming industry. Studies have estimated that gastrointestinal nematode infections lead in loses that exceed £84 million *per annum* in the UK industry alone (Nieuwhof and Bishop, 2005), with *T. circumcincta* being the major contributor. These loses are associated with the reduced productivity of the animals and the cost of the treatment. However, the cost of sub-clinical infections, which are likely to be significant, is not included in the above figures (Nieuwhof and Bishop, 2005). The impaired productivity of the animals caused by the parasite has been correlated not only with changes in feed intake, but also with alterations in gastrointestinal function, protein, energy and mineral metabolism, and body composition (Fox, 1993, Fox, 1997). Reduced feed intake is an important factor in the pathogenesis of the disease and it has been associated with elevated blood gastrin levels and with a reduced rate of ingesta movement through the digestive track (Fox *et al.*, 1989). The alterations in gastrointestinal function include: reduced motility which decreases the rate of passage of ingesta, changes in secretions such as reduced gastric acid secretion and increased blood pepsinogen and gastrin levels and impaired digestion and absorption of nutrients (Fox, 1993, Fox, 1997). These changes may be triggered by the parasite's excretions/secretions (ES), ultimately resulting in a protein losing gastropathy (Simpson, 2000).

1.2.3 Control of the parasitism

1.2.3.1 Anthelmintic drugs and anthelmintic resistance

Control of *T. circumcincta* is largely based on the administration of an effective anthelmintic drug (Scott, 2007). These drugs interact with vital parts of the parasites and compromise their biological functions (Kohler, 2001). The three major anthelmintic drug classes are the benzimidazoles, the macrocyclic lactones and the imidazothiazoles-tetrahydropyrimidines (Kaplan, 2004). Recently, two more anthelmintics were introduced, i.e. monepantel (Kaminsky *et al.*, 2008b) and derquantel (Little *et al.*, 2010). The former belongs to the amino-acetonitrile derivatives (AADs) class (Kaminsky *et al.*, 2008a) and the latter to the spiroindoles drug class (Little *et al.*, 2010).

Benzimidazoles cause the parasites' death by altering the ultrastructure of the parasites' intestinal cells (Borgers and De Nollin, 1975). In particular, the drug binds to the beta-subunit of the tubulin protein and inhibits its polymerisation (Kohler and Bachmann, 1981, Lacey, 1988, Sackett and Varma, 1993). This inhibits the development of microtubules which leads to the inappropriate release of digestive enzymes in the intestinal cells' cytoplasm causing the parasites' death (Kohler, 2001, Shompole *et al.*, 2002). Imidazothiazoles-tetrahydropyrimidines are nicotinic acetylcholine receptor agonists that interact with the nicotinic acetylcholine receptors (nAChR) located at the surface of somatic muscle cells of nematodes (Evans and Martin, 1996, Martin *et al.*, 1996). The drug binds to nicotine acetylcholine receptors on the surface of the somatic cells and causes excitation which leads to spastic paralysis of the parasite's muscles and worm expulsion (Evans and Martin, 1996, Martin *et al.*, 1996, Martin, 1997). The macrocyclic lactones cause flaccid paralysis of the somatic muscles by targeting the parasite's glutamate- (GluCl) and GABA-gated (GABACl) chloride channels (Hotson, 1982, Martin *et al.*, 1996). This leads to irreversible chloride ion currents, polarisation of the cell membrane, paralysis and finally worm expulsion (Hotson, 1982, Martin *et al.*, 1996). Moreover, they are considered to reduce pharyngeal pumping which leads to starvation of the parasite (Hotson, 1982, Geary *et al.*, 1993, Martin *et al.*, 1996). AADs have a different mode of action than the anthelmintics mentioned earlier, which involves a unique and

nematode specific clade of nAChR subunits as described by Kaminsky *et al.* (2008a). These drugs cause hypercontraction of the body wall muscles which leads to paralysis, spasmodic contractions of the anterior portion of the pharynx and, finally, death of adult nematodes (Kaminsky *et al.*, 2008a). Regarding derquantel, there are not many studies conducted to examine the mode of action. Thus far, it is known that derquantel interferes with B-subtype nAChR and leads to flaccid paralysis of nematodes (Robertson *et al.*, 2002, Epe and Kaminsky, 2013).

Parasitic nematodes over the years have developed anthelmintic resistance and this has enabled them to escape the mechanism of action of the anthelmintic drugs. Briefly, the administration of a new anthelmintic drug initially kills the susceptible parasites which are the majority of the population. However, the parasites that survive the treatment (resistant) are small in numbers at first but their numbers increase exponentially in the face of continued drug usage (Prichard, 1994). The major factor that aided the development of resistance is the high level of the parasites' genetic diversity due to the large populations and the rapid rates of nucleotide sequence evolution in combination with host movements (Blouin *et al.*, 1995, Anderson *et al.*, 1998). The former facilitated parasite populations to acquire resistance to anthelmintics and the latter promoted to spread the resistant genes (Kaplan, 2004). Anthelmintic resistance is a phenomenon that was initially reported in the late 1950s for the drug phenothiazine (Drudge *et al.*, 1957, Drudge and Elam, 1961). After this first report, the 'modern' anthelmintic drugs were introduced and the combination of their high efficacy with their low cost led to the excessive use of these drugs for the control of parasitic diseases: this approach has proven to be 'flawed' since widespread resistance has developed. There are many reviews describing the development of anthelmintic resistance over the decades, i.e. for the benzimidazoles in the 1960s, the imidazothiazoles-tetrahydropyrimidines in the 1970s, the macrocyclic lactones in the 1980s and the first cases of multiple anthelmintic drug resistance in the same decade (Coles, 1986, Kaplan, 2002, Kaplan, 2004, Wrigley *et al.*, 2006, Sargison, 2011). The introduction of monepantel in 2008 (Kaminsky *et al.*, 2008b) and derquantel in 2010 (Little *et al.*, 2010) helped to fill the gaps created in the control management of parasitic helminths by the development of

multiple resistant parasites. Recent studies have shown that *T. circumcincta* and *Trichostrongylus colubriformis* have developed resistance to monepantel (Scott *et al.*, 2013). Although there are not any reports of resistance against derquantel, history has shown that it is most likely that in the long-term the parasites will develop resistance against this anthelmintic and the commercial sheep farms would have to face the same problem as today.

The genetic mechanisms of the development of resistance have been studied and reviewed by many (Prichard, 1990, Jackson and Coop, 2000, Wolstenholme *et al.*, 2004, Von Samson-Himmelstjerna *et al.*, 2007). In general, anthelmintic resistance may result after a molecular change in the target that reduces or ablates drug binding or action, and/or after a change in the parasite's metabolism that removes or inactivates the drug before it is effective, and/or after a change in the parasite that does not allow the drug to reach its site of action and/or after an increase of the parasite's target genes that allows it to counterbalance the effect of the drug administered (Wolstenholme *et al.*, 2004). Benzimidazole resistance has been found to occur after mutations in two β-tubulin genes that inhibit the binding of the drug with its target (Kwa *et al.*, 1995). Studies have shown that the membrane of resistant nematodes has reduced binding affinity at a low affinity site for a levamisole analogue (Sangster *et al.*, 1998); whilst macrocyclic lactones resistance has been associated with mutations in the target genes (GluCl and/or GABA-R) or with overexpression of P-glycoproteins (Dent *et al.*, 2000, Kerboeuf *et al.*, 2003); however, the key mechanism of resistance to macrocyclic lactones in nematodes remains to be ascertained.

The development of anthelmintic resistance cannot be stopped, however it can be delayed by reducing the selection pressure for resistance by optimising conditions for the maintenance of refugia (van Wyk, 2001). Parasites in refugia are a sub-population of nematodes that have not been exposed to the drug treatment (van Wyk, 2001). Refugia comprises the larvae on pasture derived from untreated members of a herd. A lot of research has focused on the development of farm management practices which can be used to optimise drug use and minimise the proportion of the parasite population exposed to anthelmintics. Targeted treatments

(TT) and targeted selective treatments (TST) are refugia-based strategies which are thought to decelerate the decline in efficacy of an anthelmintic and, at the same time, maintain the animal productivity (Kenyon and Jackson, 2012). With TT a group of animals within a flock is treated based on infection markers, such as faecal egg counts (FEC), whilst with TST the treatment is targeted to individual animals based on parasitological (e.g. FEC), pathophysiological (e.g. diarrhoea score) and/or production (e.g. weight gain) markers (Kenyon and Jackson, 2012). It has been shown that selected treatment of animals based on their infection level does not have a negative effect on productivity (Hoste *et al.*, 2002), and also increases the proportion of parasites in refugia which are susceptible to the anthelmintic and, thus, reduces the selection pressure for anthelmintic resistance (Wolstenholme *et al.*, 2004). Finally, the SCOPS (Sustainable Control of Parasites in Sheep; http://www.scops.org.uk/) group was formed in the U.K. recently. Its aims are "to develop sustainable strategies for parasite control in sheep, facilitate and oversee the delivery of these recommendations to the industry and ensure that new research and development is incorporated to refine and improve advice given to the sheep industry" (http://www.scops.org.uk/). In this website, management actions are suggested to reduce the level of parasite infection and simultaneously the dependence on the anthelmintic drugs.

1.2.3.2 Grazing management

Specific grazing management practices can help towards the control of the helminths by creating 'safe pastures' and limiting the interaction between the parasites and their host (Brunsdon, 1980). This can be achieved by leaving pastures ungrazed for a period of time or by alternating cattle (or other species or practices) and sheep grazing (Brunsdon, 1980). The successful application of such an approach depends on the local epidemiological conditions which affect the biology of the dominant parasitic species (Barger, 1999). Nevertheless, this approach is not easy to apply. First of all, small commercial farms may not have the necessary pasture available in order to leave a part of it unused and farms that breed only sheep or cattle have limited options to alternate grazing. Another limiting factor is that the

animals should be treated with an anthelmintic before their introduction to the 'clean' pasture to prevent re-infection of the pasture (Leathwick *et al.*, 1995). This could lead to selection of resistant parasites and contamination of the 'safe' pasture exclusively with resistant strains of parasites (Leathwick *et al.*, 1995). Finally, it is believed that successful grazing management could lead the parasites to select other traits beneficial to them, i.e. reduced host specificity or increased longevity of the infective stage of the parasites (Barger, 1997).

1.2.3.3 Dietary supplementation

Detailed research has been conducted to study the relationship between the host's nutritional status and its immune response to gastrointestinal nematodes (Coop and Kyriazakis, 2001, Athanasiadou and Huntley, 2008). High protein intake and supplementation of the host diet with tannins have been shown to affect the infection status and the immunity (Athanasiadou *et al.*, 2000, Valderrabano *et al.*, 2002). In *T. circumcincta* infected lambs, it has been shown that an increase of protein supply not only improved carcass quality, but also enhanced the development of resistance to nematode infection, by decreasing female parasite size and fecundity (Valderrabano *et al.*, 2002). Another study showed that calves with low protein intake had higher faecal egg counts (FEC) than calves with high protein intake after trickle infection with the related nematode, *Ostertagia ostertagi* (Mansour *et al.*, 1992). In addition, a higher protein intake has been shown to have a beneficial effect on the immune system of ewes during the periparturient period when a relaxation in immunity to nematodes is observed (Houdijk *et al.*, 2000, Sakkas *et al.*, 2012). For example, high protein intake by ewes during the last part of gestation aids not only with the production immunoglobulins, mast cells etc., but also has been proposed to compensate for the protein loss caused by parasitic gastroenteritis (Houdijk *et al.*, 2000). The only drawback of this approach is economic as the expense of a high protein ration may outweigh the parasitological benefits.

Other studies have indicated that plants rich in condensed tannins (CTs) have anti-nematode properties. For example, lambs supplemented with lotus or *Quebracho*

(both rich in CTs) extracts and then challenged with *T. circumcincta* and *T. colubriformis*, respectively, had lower FEC and worm burdens compared to lambs with no supplement in their diet (Niezen *et al.*, 1998, Athanasiadou *et al.*, 2000). It has been shown subsequently that CTs interact with the dietary proteins; they form complexes and improve nutrient uptake as they dissociate later in the intestinal track (Coop and Kyriazakis, 2001). The precise mode of action of tannins has not been revealed yet, however it has been suggested that they may have a direct anthelmintic effect by affecting the biology of the parasites (Bahuaud *et al.*, 2006, Brunet *et al.*, 2007, Brunet *et al.*, 2008, Brunet *et al.*, 2011); or that their anthelmintic properties rely on the improvement of the nutrient uptake (Coop and Kyriazakis, 2001, Hoste *et al.*, 2012).

1.2.3.4 Nematophagous fungi

Nematophagus fungi are carnivorous fungi which trap and digest nematodes. Some species live inside the nematodes (endozoic), others trap the nematodes by glue traps or rings whilst others have the ability to attack the egg stage of the parasites (Waller and Larsen, 1993). Studies have shown a reduction of infective larvae in the faeces in animals administered *Duddingtonia flagrans* spores *per os* as this species can survive passage through the gastrointestinal tract and then feed on larvae in the faecal pat. This effect has been demonstrated in different hosts and with *T. circumcincta* (Paraud and Chartier, 2003); *T. colubriformis* (Paraud and Chartier, 2003); *H. contortus* (Chandrawathani *et al.*, 2002); and *O. ostertagi* (Gronvold *et al.*, 1993). *Harposporium anguillulae* are endozoic fungi which live in the faeces, produce and release crescent-shaped conidia which are ingested by nematode larvae and cause lysis of the parasites' internal organs (Charles *et al.*, 1996). It has been shown that when *H. anguillulae* was added to coprocultures, the majority of the *H. contortus* L3 population was killed (Charles *et al.*, 1996). More recent studies have shown that *D. flagrans* is suitable for reducing the numbers of infective larvae in the pasture (Chartier and Pors, 2003, Paraud and Chartier, 2003), whilst others suggest that the same fungus had no obvious effect on the larvae numbers (Epe *et al.*, 2009). A drawback of using nematophagous fungi as an anthelmintic control method is that

they only affect the worms residing in the faecal pat and not the ones that moved on pasture. Moreover, the climatic conditions affect the survival of the fungi; for example, the activity of nematophagous fungi depends on the time of the grazing season and, hence, different fungi species must be used in different seasons and environments (Waller and Larsen, 1993).

1.2.3.5 Genetic selection of the host

Genetic selection could improve host resistance to nematode parasites (Woolaston and Baker, 1996). It has been shown that there is great variability among and within breeds of animals in both resistance and resilience to nematode infection, which could translate as an effective immune response that would suppress parasite establishment or a stable production level in parasitized animals, respectively (Woolaston and Baker, 1996). 'Exotic' breeds of sheep (for example, Red Maasai, Florida Native, Barbados Blackbelly sheep) have been found to be more resistant to nematode infections than standard European breeds, such as Merino and Rambouillet (Windon, 1996). However the production traits (i.e. wool and meat) of the former breeds are not as desirable (Windon, 1996). The observed variation in resistance among and within the breeds is due to several heritable factors (Windon, 1996). For example, *H. contortus* resistance has been correlated with increased antibody-, cellular- and effector-responses (Gill *et al.*, 1993a, Gill *et al.*, 1993b, Gill, 1994); whilst, *T. circumcincta* resistance has been linked to IgA responses (Stear *et al.*, 1995). Breeding programs were examined, where resistant individuals of a flock were selected based on FEC with encouraging results for both *T. circumcincta* and *H. contortus* (Bouix *et al.*, 1998, Kahn *et al.*, 2003).

1.3 Host immunity

Protective immunity against *T. circumcincta* can be induced after recurring trickle infections over a period of four to eight weeks (Smith *et al.*, 1983b, Seaton *et al.*, 1989). The host's immune response targets L3, L4 and adult stages of the

parasite (Miller, 1996). Many studies have been conducted in order to decipher the mechanisms that lead to immunity against gastrointestinal (GI) helminths, including *T. circumcincta*. Immunity is multifaceted and involves a combination of local hypersensitivity, antibody and inflammatory responses (Smith, 1999). A common finding in GI helminthoses is increased concentrations of mucosal mast cells and globule leukocytes locally (Balic *et al.*, 2000b). Histamine, proteases, leukotrienes and cytokines are released after degranulation of mast cells and help in parasite expulsion (McKay and Bienenstock, 1994). In addition, an increase of the pepsinogen content in the gastric lymph has been proposed as a sign of hypersensitivity reactions in *T. circumcincta* infections (Smith *et al.*, 1984). The layer of mucus that coats the gastrointestinal tract is another factor that aids in host resistance to GI helminths. This mucus not only has a thick and viscous texture that makes movement of the parasites more difficult, but may also contain specific antibodies, leukotrienes, histamine, prostaglandins and amines that inhibit the parasite development and migration (Douch *et al.*, 1983, Claerebout *et al.*, 1999). Douch *et al.* (1983) have shown that the anthelmintic properties of the mucus were not species specific. In particular, mucus from sheep previously infected with *T. colubriformis* was shown to inhibit migration of *H. contortus, Nematodirus spathinger* as well as *T. circumcincta* (Douch *et al.*, 1983).

Another common finding in many helminth infections in sheep is eosinophilia, which has been associated with resistance to gastrointestinal nematode infections (Charleston, 1965, Dawkins *et al.*, 1989, Stevenson *et al.*, 1994). Furthermore, the eosinophilia response affects the larval rather than the adult stage of the parasite and in the absence of contact between the eosinophils and the larvae, the establishment of the parasite is not affected (Balic *et al.*, 2000b). Stevenson *et al.* (2006) studied the eosinophil response after primary and secondary infection with *T. circumcincta*. The results of the study showed that, in helminth-free sheep, 10 days after primary infection, the worm burdens were high and the tissue eosinophilia was mild; whilst 21 days after primary infection the results were inversed with lower worm burdens and higher eosinophilia (Stevenson *et al.*, 1994). After secondary infection, eosinophilia peaked 2 dpi and remained at high levels until 10 dpi, when the animals

were sacrificed (Stevenson *et al.*, 1994). Furthermore, a more recent study has shown that eosinophils interact directly with L3 *H. contortus in vivo* and that they can damage or even kill the larvae (Balic *et al.*, 2006). *T. circumcincta* larvae develop inside the gastric glands of the abomasum which allows the larvae to develop in the absence of eosinophils and emerge from the glands near maturity, when they are less susceptible to the damage caused by the eosinophils (Balic *et al.*, 2000b).

Lymphocytes are also associated with the development of immunity to GI nematodes. There are three types of lymphocytes, which include natural killer cells (NK cells), T-cells and B-cells. Similarly to what was mentioned before with eosinophilia, the increase of lymphocytes has been correlated with the larval rather than the adult stage of the parasites (Balic *et al.*, 2000b). Studies have shown that after primary infection with *T. circumcincta*, there was an increase of lymphocytes and lymphoblasts in gastric lymph from the 8th dpi until the 21st dpi (Smith *et al.*, 1983a). Similar studies with *H. contortus* and *O. ostertagi* have shown an increase of T- and B-cells in the abomasal lymph nodes and tissues during the first week after the primary infection (Balic *et al.*, 2000a, Balic *et al.*, 2000b). An increase of lymphocytes was observed within 5 days after a challenge infection of previously infected sheep with *T. circumcincta* (Smith *et al.*, 1983b). Another study showed that when lymphocytes from previously infected sheep were transferred intravenously to monozygotic twin helminth-free sheep, partial immunity was transferred to parasite-naive sheep (Smith *et al.*, 1986). The latter findings support the rationale that vaccination could be an alternative control method against *T. circumcincta*.

Cytokines are a large group of proteins that play an important role in immune cell signalling and include, among others, chemokines, interferons (IFN) and interleukins (IL). In *T. circumcincta* infections, a significant up-regulation of IL-4, IL-5, IL-13, IL-1β, IL-6, IL-10, IL-18, transforming growth factor-β_1 (TGFβ_1) and tumour necrosis factor-α (TNFα) was found by the 5th dpi of previously infected animals compared to naïve sheep (Craig *et al.*, 2007). This up-regulation was correlated with a reduction in worm burdens in previously infected- compared to naïve animals (Craig *et al.*, 2007). The expression levels of IL-2 and IFN-γ were not significantly different between the groups of animals (Craig *et al.*, 2007).

Furthermore, a comparison between the abomasal mucosa and the gastric lymph node in naïve and previously infected sheep showed that the abomasal mucosa is a major source of cytokines during helminth infection in previously infected animals (Craig *et al.*, 2014). The expression pattern of the cytokines in animals infected with other nematode infections is similar to the aforementioned response. Almeria *et al.* (1997) have analyzed the expression of cytokine mRNA in mucosal lymphocytes and abomasal lymph nodes after a primary infection with O. ostertagi in cows. The results showed an increase of IL-4, IFN-γ and IL-10 mRNA before the 10th dpi for the former two and after the 10th dpi for the latter (Almeria *et al.*, 1997). Similar studies have demonstrated that a primary infection of lambs with H. contortus leads to an increase of IFN-γ and IL-10 in the abomasal lymph nodes as early as 3 dpi, however they recovered to normal levels after the 5th dpi (Balic *et al.*, 2000b). IL-4 was not increased after the primary infection but there was an elevation in the abomasal lymph nodes after a challenge infection with H. contortus within 5 dpi, whilst there was an increase of IFN-γ in the tissues within 3 dpi (Balic *et al.*, 2000b). Finally, a comparison between resistant and susceptible Brazilian Somalis crossbreed sheep naturally infected by *H. contortus* showed that in the abomasal lymph nodes there was an up-regulation of IL-4 and IL-13 in the resistant animals and an up-regulation of IFN-γ in the susceptible (Zaros *et al.*, 2014).

Antibodies (immunoglobulins) are proteins which are used by the immune system to identify and neutralize invading organisms by recognizing a unique target (antigen) that originates from the foreign organism. Specific antibodies are involved in host resistance to GI nematode infections as their serum titres are higher in secondary infections compared to primary infections (Balic *et al.*, 2000b). Immunoglobulin A (IgA) is considered to be associated with the resistance to *T. circumcincta* in sheep. Studies have demonstrated negative correlations between worm length / worm fecundity and L4–specific IgA in the serum and in the gastric lymph after challenge infections (Smith *et al.*, 1983b, Smith *et al.*, 1983a, Stear *et al.*, 1995, Stear *et al.*, 1999a). Although there is a negative correlation, the precise function of IgA in parasitized animals is unclear. It has been suggested that IgA might interfere with the feeding of the parasite (Smith, 1988) or it might bind to

inflammatory cells in the mucosa and lead to the release of cytokines and other inflammatory mediators (Dubucquoi *et al.*, 1994). Immunoglobulin G (IgG) is also considered to be involved in the host immunity. Studies have shown a significant rise in anti-L3 IgG titres 3 weeks after *T. circumcincta* (Sutherland *et al.*, 1999). Furthermore, IgG has been negatively correlated with FEC in lactating ewes after secondary infection with *T. circumcincta* (Cruz-Rojo *et al.*, 2012). Studies in different nematodes have also shown an increase in the serum IgG1 and IgG2 after secondary infections of sheep and calves with *H. contortus* and *O. ostertagi*, respectively (Canals and Gasbarre, 1990, Schallig *et al.*, 1995). Finally, immunoglobulin E (IgE) has been implicated with immunity to GI helminth infections (Kooyman *et al.*, 1997). Studies in *T. circumcincta* have shown an increase of IgE in both lymph and serum in naïve and previously infected sheep (Huntley *et al.*, 1998b). However, the IgE concentration in lymph was greater than in serum which suggests that the production of the IgE was local, i.e. in the mucosa or the draining nodes (Huntley *et al.*, 1998b). Moreover, the same group demonstrated that after a challenge infection of previously infected sheep there was an IgE antibody response to L3 antigens in the gastric lymph (Huntley *et al.*, 1998a). The latter finding supports the previous suggestion that the IgE was potentially generated in the mucosa or the draining nodes.

1.4 Vaccination as an alternative control strategy and vaccine development strategies

As mentioned before, sheep are able to develop protective immunity against most GI nematodes including *T. circumcincta* and in the case of this parasite, protective immunity can be adoptively transferred from immune to worm-free lambs (Smith *et al.*, 1986). In experimentally immunized sheep, a degree of protective immunity is observed after four weeks of trickle infection with L3 and this is seen as a retardation of development of the worms in the gastric gland (Seaton *et al.*, 1989). If the animals are exposed to the parasite for 12 weeks, they have a higher degree of immunity both to larval development and to incoming parasites which appear to fail

to establish in the abomasal wall (Seaton *et al.*, 1989). However, the level of immunity that is induced following trickle infection depends on several variables, such as the age of the animal (Smith *et al.*, 1985), the genotype of the animal (Windon, 1996, Woolaston and Baker, 1996, Beraldi *et al.*, 2008) and whether or not the sheep is in the periparturient period (Barger, 1993). In particular, young sheep (4.5 months old) are less resistant to *T. circumcincta* challenge and would appear to require more time to mount sufficient protective immunity than 10 months old animals (Smith *et al.*, 1985). Moreover, immunity in ewes is compromised during parturition and early lactation (Barger, 1993).

Taking into consideration all the above, vaccination should be a feasible strategy for the future control of *T. circumcincta*. Barnes *et al.* (1995) used a mathematical model to predict the results of a potential vaccine for worm control. They suggested that vaccination is a better approach than the traditional strategies based on the administration of anthelmintic drugs because their simulations have shown that the death rate due to infection never exceeded four out of 2,000 lambs (Barnes *et al.*, 1995). Moreover, after simulating different degrees of vaccine efficacies (i.e. 60, 70, 80, 90 and 100%) with different flock responses to the vaccine (i.e. 60, 70, 80, 90 and 100%) the results have shown that vaccination would be sufficiently beneficial even when a vaccine only provided 60% protection in 80% of the flock (Barnes *et al.*, 1995). Hitherto, there were several approaches towards the development of a vaccine against helminths.

1.4.1 Radiation attenuated worms as vaccines

A strategy that has been successfully used in the past for the vaccine development against helminths was the use of radiation-attenuated worms. Such vaccines use live parasites attenuated by X-, ultraviolet- or γ-radiation. The attenuated worms cannot reproduce in the host nor cause substantial pathology; however they can stimulate a protective immune response in the host (Miller, 1964). This approach was used to develop a vaccine against the cattle lungworm, *Dictyocaulus viviparus* (Bovilis® Huskvac, Intervet, UK Ltd), which has been

commercially available since the 1960s (Jarrett *et al.*, 1958, Smith, 1999, McKeand, 2000). This vaccine is administered *per os* and contains 40 krad-irradiated larvae that stimulate an immune response in the mesenteric lymph nodes, however they do not develop to the adult stage that causes the disease (Jarrett *et al.*, 1958). Radiation attenuated parasites were also used to develop an effective vaccine against *Ancylostoma caninum* in the USA during the 1970s (Miller, 1971). This vaccine used X-irradiated *A. caninum* L3 and was found to stimulate over 90% protection against challenge infection. Nevertheless, the establishment of a small number of the irradiated worms in the intestine left dog owners unsatisfied and as a result the product was withdrawn (Miller, 1978). The same principles have been used to try and develop vaccines for GI nematodes. Although immunity develops relatively swiftly for *D. viviparus* in calves and *Dictyocaulus filaria* in lambs, immunity to GI nematodes is acquired at a slower rate (Smith, 1999). Live attenuated vaccines for *H. contortus* and *T. colubriformis* were found to induce protective immunity in mature sheep under experimental conditions (Jarrett *et al.*, 1961, Windon *et al.*, 1984). The effect of this approach has also been examined for vaccination against *T. circumcincta* (Smith *et al.*, 1982); the results indicated that after two doses of irradiated *T. circumcincta* larvae, immunised sheep had significantly less adult worms after challenge (Smith *et al.*, 1982). Moreover, when irradiated larvae were removed 7 days before the challenge by treatment with anthelmintic, sheep were protected against subsequent challenge (Smith *et al.*, 1982).

Irradiated vaccines also have a number of practical drawbacks; for example, the use of live worms makes the product unstable which reduces its shelf-life, and requires a cold chain for distribution (McKeand, 2000). Furthermore, there are also the ethical and financial implications of producing the parasites for generation of the vaccines as donor animals are required to prepare the biological material (McKeand, 2000).

1.4.2 'Hidden' antigens as vaccine targets

The 'hidden' antigens are proteins expressed inside the parasite such as on the luminal surface of the intestine. These molecules are thought not to be released into the host and do not stimulate an immune response during natural infection (Smith, 1993, Smith, 1999). The hidden antigen vaccine development approach has been used to target haematophagous parasites mostly. The rationale behind this is that animals are immunized with hidden gut membrane proteins and when the blood-feeding parasites feed, they ingest antibodies, which bind to and damage the intestinal proteins. This has proved a successful approach in vaccination against the blood-feeding nematode, *H. contortus*. A number of gut membrane proteins and protein complexes have been isolated from this parasite and were found to cause more than 80% reduction in FECs and more than 50% reduction in worm burdens in experimental trials (Jasmer *et al.*, 1993, Smith *et al.*, 1993c, Smith and Smith, 1993, Smith *et al.*, 1994). H11, a microsomal aminopeptidase expressed in *H. contortus*' intestinal microvillar surface, was successfully used to immunize sheep and led to significant protection against challenge infection (Smith *et al.*, 1993c, Andrews *et al.*, 1995, Newton *et al.*, 1995). Other examples of *H. contortus* hidden antigens that were shown to give significant protection in lambs are H-gal-GP (*Haemonchus* galactose containing protein complex) which contains a number of proteases (Smith and Smith, 1996) and TSBP (thiol sepharose binding proteins) which are enriched with cysteine proteases (Knox *et al.*, 1999). Recently, a vaccine was developed at Moredun Research Institute by Dr. David W Smith and Dr. George Newlands to control *H. contortus* and this vaccine is commercialized in Australia. The major antigenic components of this vaccine have been shown to be the well characterized H-gal-GP (Smith *et al.*, 1999) and H11 (Smith *et al.*, 1993c, Smith *et al.*, 1997) which are extracted and purified from adult *H. contortus* from donor sheep and are used in their native form. The commercial name of the vaccine is 'Barbervax' and this will be the first commercial vaccine for a GI nematode in ruminants.

Although this approach was fruitful for *H. contortus*, it has not proved successful for non blood-feeding parasites, such as *T. circumcincta*. For example, Smith *et al*. (2001) conducted two vaccine trials using native gut membrane

glycoproteins from *H. contortus*, in the first trial, and *T. circumcincta*, in the second trial, as antigens. The results demonstrated that immunization of lambs with *H. contortus*' gut membrane glycoproteins provided significant protection against *H. contortus* (99% reduction in egg counts) but not against *T. circumcincta* challenge when compared to challenge control sheep (Smith *et al.*, 2001). Similarly, immunization with *T. circumcincta* gut membrane glycoproteins conferred a significant protection against *H. contortus* (54% reduction in egg counts) but not against *T. circumcincta* challenge (Smith *et al.*, 2001). It was proposed from these studies, that *T. circumcincta* ingested insufficient antigen specific antibody for this type of vaccination approach to work.

1.4.3 Extracellular microvesicles as novel vaccine targets

Cell communication in multicellular organisms occurs via extracellular molecules (nucleotides, lipids, short peptides, proteins) and extracellular microvesicles (EMVs) that are released in the micro-environment (Thery, 2011). The molecules bind to receptors of other cells and alter the physiological state of the cells they encounter, whilst the EMVs, which contain proteins, lipids and nucleic acids, affect the recipient cells in more complex ways (Thery, 2011). EMVs include exosomes, shedding microvesicles and apoptotic bodies, which differ primarily in size and how they are generated (Mathivanan *et al.*, 2010). Exosomes are small membranous vesicles of endocytic origin, approximately 50 – 100 nm in diameter (Thery *et al.*, 2006), and were initially described in the 1980s (Pan and Johnstone, 1983). They originate from large multivesicular bodies (MVBs) and are released to the extracellular space after fusion of the MVBs with the plasma membrane (van Niel *et al.*, 2006, Simpson *et al.*, 2009). Shedding microvesicles are larger (100 - 1000 nm) and are formed by direct blebbing of the plasma membrane (Cocucci *et al.*, 2009). Apoptotic bodies are larger membrane vesicles (1-4 μm) that are formed during apoptosis (Hristov *et al.*, 2004). The precise nature and function of exosomes were studied more intensively over the past decade and this has been reviewed by many (Couzin, 2005, Simons and Raposo, 2009, Mathivanan *et al.*, 2010, Bobrie *et*

al., 2011, Thery, 2011, Montaner *et al.*, 2014). The growth in this field of research is due to the discovery of the important roles that exosomes may play in intercellular signalling and cell-cell communication.

There were two major breakthroughs, which suggested that exosomes are specific functional cell products. Studies have shown that exosomes derived from different cell types not only include proteins which belong to a few specific families (Thery *et al.*, 1999, Thery *et al.*, 2001, Mathivanan and Simpson, 2009, Mathivanan *et al.*, 2012, Simpson *et al.*, 2012), but also contain microRNA (miRNA) and mRNA which could be translated in target cells (Valadi *et al.*, 2007). The studies that initially indicated the importance of exosomes were from the field of immunology where it was demonstrated that not only B-lymphocytes (Raposo *et al.*, 1996), but also dendritic cells (Zitvogel *et al.*, 1998), secrete exosomes which contain molecules that affect immune responses (Andreola *et al.*, 2002, Thery *et al.*, 2002). The results of these studies suggested a potential role of exosomes in intercellular communication in the immune system. Other potential functions, in addition to the activation and/or suppression of the immunological responses, include: the transfer of pathogenic proteins, tissue repair and neural communication (Couzin, 2005, Simons and Raposo, 2009).

The first report of EMVs in helminths was in the 1960s in *Fasciola hepatica* where they were characterized as "small membrane-limited vesicles" (Threadgold, 1963). A subsequent study in the 1980s in *Echinostoma caproni* also described the existence of "membrane bound vesicles" (Andresen *et al.*, 1989). A recent study has shown that the same trematode parasites (*F. hepatica* and *E. caproni*) actively release exosomes in their micro-environment (Marcilla *et al.*, 2012). Other examples of organisms that were found to release exosomes include, among others: the nematodes, *Caenorhabditis elegans* (Liegeois *et al.*, 2006) and *Heligmosomoides polygyrus* (Buck *et al.*, 2014); the cestode *Echinococcus multilocularis* (Eger *et al.*, 2003); and the protozoan parasites *Leishmania donovani* (Silverman *et al.*, 2010) and *Toxoplasma gondii* (Pope and Lasser, 2013). Marcilla *et al.* (2012) showed that intestinal rat cells actively take up exosomes secreted by *F. hepatica* and *E. caproni*. A recent study demonstrated that small RNAs contained in exosomes secreted by *H.*

polygyrus regulate genes of the innate immune system (Buck *et al.*, 2014). These observations support the belief that the exosomes play an important role in the host-parasite interface, and thus, it has been suggested that exosomes could be potential candidates for vaccine and/or drug interventions (Marcilla *et al.*, 2012).

1.4.4 Excretory/Secretory molecules and surface antigens as vaccine targets

Excretory/Secretory (ES) products comprise molecules that are released or excreted by helminths and include a variety of enzymes such as proteases, acetylcholinesterases, superoxide dismutases, as well as potential immunomodulators (Knox, 2000, Smith *et al.*, 2009). In *T. circumcincta*, the ES products are released in a developmental stage-specific manner from the body surface or by excretory/secretory glands which are thought to be located at the anterior part of the worms (Knox, 2000). It is suggested that these molecules might play a critical role in the parasite's survival in the host by helping them evade or modulate the host immune response, penetrate host tissues and to feed (Knox, 2000). In this respect, parasite establishment could be prevented by vaccinating with an essential ES product and stimulating an immune response which subsequently blocks its function.

The most 'popular' vaccine development strategy nowadays attempts to identify susceptible and accessible molecules either in the ES products or on the surface of the parasites (Smith, 1999). A common approach for vaccine development based on the ES products has three steps. First, is the screening of potential protective fractions in preliminary protection trials; then, potential protective components are purified; and, finally, functional recombinant version of the candidate protein are generated and tested in vaccine trials (Smith, 1999). With respect to *T. circumcincta,* in previously infected sheep (more than 6 months-old), protective immunity manifests within the first 48 hours post challenge as a reduction in the worms' ability to establish and develop leading to a reduction in faecal egg excretion and overall worm burden (Stear *et al.*, 1995, Stear *et al.*, 1999a, Halliday *et al.*, 2007, Halliday *et al.*, 2009). Bearing the aforementioned facts in mind, the quest

for potential vaccine candidates against *T. circumcincta* has focused on the ES products released by *T. circumcincta* during the early stages of infection, because these are the most critical stages in the host/parasite interaction in the early stages of developing immunity (Smith *et al.*, 2009).

Many studies have been conducted to identify the best vaccine candidates in the L3 and L4 *T. circumcincta's* ES products. To date, the principal ES products of *T. circumcincta* that have developed to vaccine targets include: an immunogenic cathepsin F (Tci-CF-1), which is the most abundant molecule in the ES products of the L4 stage of the parasite and is a target of local IgA in immune sheep (Redmond *et al.*, 2006); a surface associated antigen (Tci-SAA-1), which is an immunogenic homologue of a protective antigen from *A. caninum* and appears to be only expressed in L3 (Zhan *et al.*, 2004, Nisbet *et al.*, 2009); an activation-associated secreted protein (Tci-ASP-1) and an astacin-like metalloproteinase (Tci-MEP-1), both of which are dominant ES molecules released from L4 and are also targets of early IgA responses in previously infected sheep (Smith *et al.*, 2009, Nisbet *et al.*, 2010b); a macrophage migration inhibitory factor-like molecule (Tci-MIF-1) (Nisbet *et al.*, 2010a), a calcium-activated apyrase (Tci-APY-1) (Nisbet *et al.*, 2011) and a TGFβ homologue (Tci-TGH-2) (McSorley *et al.*, 2010), all of which could be potentially immunosuppressive. Furthermore, there are many studies in other parasitic helminth species that define ES products are vaccine candidates. For example, Matthews *et al.* (2001) tried to develop a vaccine for *D. viviparus*, which would be more stable than the current commercially available irradiated larval vaccine. Their search was focused on adult ES products, however, immunization of calves with whole adult ES products or with a recombinant form of the secreted enzyme, acetylcholinesterase (AChE), in the context of Freund's incomplete adjuvant did not result in significant levels of protection (Matthews *et al.*, 2001). Investigating the ES products of *A. caninum* led to the discovery of *Ancylostoma* secreted proteins or activation-associated secreted proteins (ASPs), which have proved to be promising vaccine candidates (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999). These proteins have been characterized in detail at the molecular level (Zhan *et al.*, 2003) and will be described in more detail in Section 1.5. Another example is the vaccination of calves with

fractions of *O. ostertagi* adult ES products, which induced significant levels of protection compared to unvaccinated animals (Geldhof *et al.*, 2002, Meyvis *et al.*, 2007).

As mentioned above, the final and most critical step for vaccine development is to produce and test a functional recombinant version of the potential candidate(s). Recombinant protein expression bypasses the expense of the production of native antigenic material by synthesising the target antigen economically and in large quantities (Knox *et al.*, 2001). Bacterial expression systems were used in vaccine trials against parasitic nematodes with poor results (Emery, 1996). Differences in conformation and glycosylation result in recombinant proteins expressed in bacterial expression systems to be different from the native versions and this has been proposed to be the main reason for there being so many failures with recombinant nematode vaccines (Knox *et al.*, 2001). Other expression systems that have been used include yeast, insect cells and the free-living nematode, *C. elegans*, and these might address the issues, primarily correct post-translational processing, associated with bacterially expressed recombinant proteins. Despite the previous failures to generate an effective recombinant sub-unit vaccine for ruminant GI nematodes, a combination of eight recombinant proteins derived from *T. circumcincta* have been shown on two occasions to stimulate significant levels of protection against challenge in vaccinates compared to control lambs (Nisbet *et al.*, 2013). The proteins which were used in their recombinant form included: Tci-CF-1, Tci-SAA-1, a 20 kDa protein of unknown function (Tci-ES20), Tci-MEP-1, Tci-MIF-1, Tci-APY-1, Tci-TGH-2 and Tci-ASP-1 (Nisbet *et al.*, 2013).

1.5 Activation-associated Secreted Proteins in parasitic nematodes

Evolution has helped the parasites to develop invading strategies to establish in their hosts. They excrete or secrete a variety of proteins and other molecules that have been shown to have specific functions at the host/parasite interface, for example, evasion of the host immune response, initiation and maintenance of the

infection (Schmid-Hempel, 2008). As mentioned above, predominant and immunogenic ES proteins of nematodes include a group of proteins collectively termed activation-associated secreted proteins (ASPs).

1.5.1 Features of ASPs

ASPs are members of the SCP/Tpx-1/Ag5/PR-1/Sc7 family (SCP/TAPS; Pfam accession number: PF00188), a group of evolutionary-related secreted proteins and have been reviewed by many (Cantacessi *et al.*, 2009, Chalmers and Hoffmann, 2012). The molecules of this family include: rodent sperm-coating glycoproteins (SCP), which are involved in the maturation of the sperm (Jalkanen *et al.*, 2005); mammalian testis specific proteins (TPX) (Kasahara *et al.*, 1989); glioma pathogenesis-related protein (Murphy *et al.*, 1995); venom allergen 5 proteins from vespid wasps and venom allergen 3 proteins from fire ants, which are involved in the allergic reaction caused by insect bites (Lu *et al.*, 1993); plant pathogenesis proteins of the PR-1 subfamily, which are involved in the responses to infections and stressful stimuli (van Loon *et al.*, 2006). All of these family members belong to the cysteine-rich secretory protein (CRISP) superfamily (Chalmers *et al.*, 2008). It is suggested that the SCP/TAPS proteins have a common primary structure (Figure 1. 3), which is as follows: the SCP domain (InterProScan: IPR014044), which follows on from the signal peptide region and forms the main part of the protein. There is also a cysteine-rich secreted protein (CRISP) domain (InterProScan: IPR013871) in the C-terminus region (Cantacessi *et al.*, 2009). The CRISP domain in mammals and reptile venoms contains 10 conserved cysteine residues, whilst this part in invertebrates (i.e. parasitic helminths) is shorter and contains four to six cysteine residues (Yatsuda *et al.*, 2002). It has been suggested that the SCP domain acts as a Ca^{+2}-chelator in various signalling processes (Fernandez *et al.*, 1997). It has also been indicated that SCP/TAPS proteins have a common secondary structure because the SCP domain contains six conserved cysteine residues four of which are involved in the formation of disulfide bridges (Fernandez *et al.*, 1997).

1.5.2 ASPs of nematodes

SCP/TAPS proteins have been isolated from various invertebrates, such as arthropods, roundworms (nematodes) and flatworms (Cantacessi *et al.*, 2009). They are commonly referred as ASPs (see below) in helminths and have been characterized as one of the largest nematode-specific groups of proteins (Parkinson *et al.*, 2004). Although they have been identified in nematodes of the clades III, IV and V (Cantacessi *et al.*, 2009), here, the focus is on the nematodes of clade V. Clade V includes the orders Strongylida, Rhabditida and Diplogasterida (Blaxter *et al.*, 1998). Parasites such as *H. contortus*, *O. ostertagi*, *T. circumcincta*, *Necator americanus*, *Nippostrongylus brasiliensis*, *A. caninum*, *Ancylostoma duodenale* and *Ancylostoma ceylanicum* belong to the Strongylida order. The Rhabditida order in Clade V also includes free-living nematodes such as *C. elegans* and *Caenorhabditis briggsae*; whilst nematodes such as *Pristionchus pacificus* belongs to the Diplogasterida order (Blaxter *et al.*, 1998). Hitherto, three types of ASPs have been described in nematodes (Figure 1. 3): [i)] long double-domain ASPs, composed of two distinct, but related, domains (Hawdon *et al.*, 1996, Schallig *et al.*, 1997b, Hawdon *et al.*, 1999), [ii)] short single-domain ASPs, which show similarity to the C-terminal of the double-domain type (Hawdon *et al.*, 1996, Schallig *et al.*, 1997b, Hawdon *et al.*, 1999) and [iii)] short single-domain ASPs, which show similarity to N-terminal of the double-domain type (Geldhof *et al.*, 2003).

In clade V, ASPs have been studied extensively in the dog hookworm, *A. caninum*. Hawdon *et al.* (1996), identified the first ASP molecule in the L3 stage of *A. caninum*. This is a double-domain ASP (Ac-ASP-1) found to be secreted in response to a host-specific signal early in infection (Hawdon *et al.*, 1996). As a result, these proteins were designated as ASPs since they were found to be associated with the activation of the parasite in the host and *in vitro* after stimulating the infective larvae with host serum (Hawdon *et al.*, 1996). Subsequently, a C-type single-domain ASP (Ac-ASP-2) was identified as a major component of *A. caninum* L3 ES products (Hawdon *et al.*, 1999). This protein was localized on the granules in the glandular oesophagus and, thus, it was suggested that it is secreted when the parasite invades the host (Bethony *et al.*, 2005). In addition, four ASPs were isolated

from adult stage *A. caninum*: Ac-ASP-3, Ac-ASP-4, Ac-ASP-5 and Ac-ASP-6 (Zhan *et al.*, 2003). Ac-ASP-3 protein was characterized as a C-type single-domain ASP, whilst the other three as double-domain ASPs (Zhan *et al.*, 2003). A transcriptomic analysis of *A. caninum* L3 after mimicking the initial phase of infection by culturing L3 *in vitro* in serum-containing medium revealed that the most abundant transcripts that were up-regulated encoded ASP molecules (Datu *et al.*, 2008). Homologue proteins to Ac-ASP-1 and Ac-ASP-2 have been identified in the L3 stage of the nematodes *A. ceylanicum* (Ay-ASP-1, Ay-ASP-2), *A. duodenale* (Ad-ASP-1) and *N. americanus* (Na-ASP-1, Na-ASP-2) (Bin *et al.*, 1999, Goud *et al.*, 2004). Hc24 is a C-type single-domain ASP which was the first ASP described in adult *H. contortus* as an oesophageal antigen (Takats *et al.*, 1995). The same protein was later isolated in the ES products of adult *H. contortus* and it was shown that it is transcribed solely in the L4 and adult stages of the parasite (Schallig *et al.*, 1997b). Furthermore, a double-domain ASP (Hc40) was described in adult *H. contortus* (Rehman and Jasmer, 1998). Two N-type single domain ASPs (Oo-ASP-1 and Oo-ASP-2) were characterized as major antigens in a host protective fraction from adult *O. ostertagi* (Geldhof *et al.*, 2003). These molecules were found to be transcribed in both L4 and adult stages of the parasite (Geldhof *et al.*, 2003). A stage-specific gene expression study of *T. circumcincta* transcripts revealed 99 expressed sequence tags (ESTs) which formed 43 contigs and encode ASP molecules (Nisbet *et al.*, 2008). These ESTs represent all three types of ASPs and the majority of them were found to be expressed in L4 but not in a transcript dataset derived from *T. circumcincta* exsheathed L3 (Nisbet *et al.*, 2008). A further study revealed Tci-ASP-1, an N-type single domain ASP, as one of several ASPs identified as immunogenic in the ES products of L4 *T. circumcincta* harvested 5 dpi (Nisbet *et al.*, 2010b). In the same study, a recombinant Tci-ASP-1 (rTci-ASP-1) was expressed in *Escherichia coli* cells and was found to bind IgA antibody from previously infected ewes.

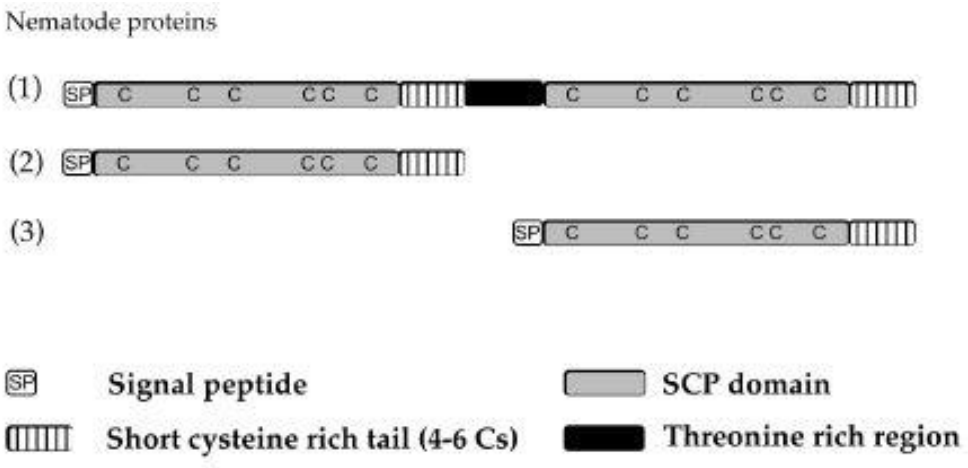


Figure 1.3. Primary structure of the SCP/TAPS proteins in nematodes. (1) Double-domain ASPs; (2) N-type single-domain ASPs; (3) C-type single-domain ASPs. Original image by (Visser, 2008).

1.5.3 ASPs as vaccine candidates

ASPs have been evaluated as vaccine candidates against a number of parasites due to their abundance in the ES products of the early parasitic stages and their immunogenicity. An ASP of *H. contortus*, Hc24, was one of the first 'single' molecules to be tested in vaccine trials (Schallig *et al.*, 1997a, Schallig and Van Leeuwen, 1997, Kooyman *et al.*, 2000). The results showed a significant protection against infection as judged by reduced worm burdens and FEC when vaccinates were compared to non-vaccinated and adjuvant control animals (Schallig *et al.*, 1997a, Schallig and Van Leeuwen, 1997, Kooyman *et al.*, 2000). A factor that complicates interpretation of these results was the presence of a 15kDa protein in the protective fraction and it is unknown which protein was actually responsible for the protective effect (Schallig *et al.*, 1997a). The function of the 15kDa protein molecule remains unknown, however molecular characterization showed that it is related to 11 kDa and 30 kDa ES products of *T. colubriformis*, which have also been found to induce significant levels of protection in guinea pigs against challenge (Schallig *et al.*, 1997b). Another finding in *Haemonchus* studies was that the protective effect was age related and, hence, variable (Kooyman *et al.*, 2000). In particular, when sheep of different ages were vaccinated, i.e. 9, 6 and 3 months-old, protection levels were

83%, 77% and 34%, respectively (Kooyman *et al.*, 2000). The authors suggested that the differences among the age groups could be due to an absence or insufficient cytokine production in the youngest lambs (Kooyman *et al.*, 2000). The same vaccine targets (i.e. Hc24 and 15kDa protein) were tested in a vaccine trial as recombinant proteins (Vervelde *et al.*, 2002). The results were inconsistent among separate trials (Vervelde *et al.*, 2002). The authors suggested that the inconsistent results might have been due to differences in the vaccine preparations (Vervelde *et al.*, 2002).

Vaccine trials have also been performed in rodent models using ASP-1 and ASP-2 of *A. caninum* (Ghosh *et al.*, 1996, Sen *et al.*, 2000, Goud *et al.*, 2004). The results of these trials have shown significant levels of protection. Nevertheless, there were some differences observed amongst trials. The studies could not concur with regard to the most appropriate vaccine target, i.e. ASP-1 (Ghosh *et al.*, 1996, Sen *et al.*, 2000) or ASP-2 (Goud *et al.*, 2004); the most appropriate adjuvant, i.e. no adjuvant (Sen *et al.*, 2000), *Corynebacterium parvum* adjuvant (Ghosh *et al.*, 1996) or Quil A (Goud *et al.*, 2004); nor the most appropriate recombinant expression system, i.e. *E. coli* (Ghosh *et al.*, 1996, Sen *et al.*, 2000) or *P. pastoris* (Goud *et al.*, 2004). In addition, the recombinant version of Ac-ASP-2 was tested in dogs and showed significant protection by reduced worm burdens and FEC in the vaccinates compared to challenge control dogs (Bethony *et al.*, 2005).

Another parasite that the ASPs have been evaluated as vaccine candidates is *O. ostertagi*. In these studies, calves were immunized with a fraction of adult ES products purified using a thiol-sepharose column (ES-thiol); the fraction was found to contain mainly Oo-ASP-1 and Oo-ASP-2, as well as cysteine proteinases (Geldhof *et al.*, 2002, Geldhof *et al.*, 2003). Vaccination induced significant protective immunity as judged by a reduction of approximately 60% in FEC compared to unvaccinated calves (Geldhof *et al.*, 2002, Geldhof *et al.*, 2004). In a subsequent study, the ES-thiol fraction was further purified and a sub-fraction containing only the two ASPs was used for a vaccine trial (Meyvis *et al.*, 2007). This immunization resulted in 47% reduction in worm burdens and more than 70% reduction in FEC compared to challenge control calves (Meyvis *et al.*, 2007). Since the results with the

native proteins were very promising, a vaccine trial was conducted with a recombinant version of Oo-ASP-1 expressed in a baculovirus expression system (Geldhof *et al.*, 2008). Alas, protective immunity against challenge was not induced by the recombinant protein, which led the authors to suggest incorrect protein folding during the expression of the protein (Geldhof *et al.*, 2008). In the closely related nematode *T. circumcincta*, Tci-ASP-1 was one of eight proteins used in recombinant forms and found to induce protective immunity in vaccinated lambs (Nisbet *et al.*, 2013). However, it is not known which of the eight vaccine targets induced immunity and whether all of them are required to induce protection.

1.5.4 Function of the ASPs

As mentioned before, many studies have shown that ASPs are promising vaccine candidates against several parasitic nematodes. Nonetheless, the precise function of these proteins remains to be elucidated. There are several suggestions regarding their potential function. In particular, it is suggested that they may have a key role in infection and transition to parasitism due to their abundance in the ES products of L3 stages (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999). Others believe that they may have a role in pathogenesis, since they are both angiogenic (Tawe *et al.*, 2000) and homologous to venom allergens from wasps (Ag5) and ants (Ag3) (Lu *et al.*, 1993); or that they may have an immunomodulatory function (Asojo *et al.*, 2005); a role in the reproduction (Geldhof *et al.*, 2003); a role in maintenance of the parasites at their host niche (Zhan *et al.*, 2003); or, finally, maintenance and/or the exit from the arrested development (Wang and Kim, 2003).

The present PhD project will, in part, focus on the ASPs not only because they are targets of the early IgA responses in previously infected sheep (Nisbet *et al.*, 2010b), but also because they have been found to provide good levels of protection against *H. contortus* in sheep (Schallig and Van Leeuwen, 1997), *O. ostertagi* in cattle(Geldhof *et al.*, 2002) and *Ancylostoma* spp. (Ghosh and Hotez, 1999). This project was initiated in an effort to shed light on the precise function of ASPs and their effect on the host immune response, the ambition being to contribute to vaccine

development not only against *T. circumcincta,* but also against other parasitic nematodes in which ASPs are dominant vaccine candidates. This objective could be addressed by developing an RNA interference platform to knock down expression of specific ASP genes *in vitro* and *in vivo,* as RNA interference has the potential to be used as a tool to define gene function in animal parasitic nematodes (APNs).

1.6 RNA interference

RNA interference (RNAi) is a reverse genetic mechanism that is triggered by gene-specific double-stranded RNA (dsRNA) and causes potent and highly specific gene silencing (Fire *et al.,* 1998). It is considered to be an ancient cellular defence mechanism against viruses and transposable elements (Matzke *et al.*, 2001, Vance and Vaucheret, 2001). RNAi was initially described in the free-living nematode *C. elegans* (Fire *et al.*, 1998), in which it has been broadly used as a tool for functional analysis of its genome (Maeda *et al.*, 2001, Kamath *et al.*, 2003). As mentioned before, the RNAi pathway is activated by gene-specific dsRNA. This dsRNA can be delivered to worms in a variety of ways including: microinjection (Fire *et al.*, 1998); feeding worms with *E. coli* bacteria that express dsRNA (Timmons and Fire, 1998); soaking worms in a dsRNA solution (Tabara *et al.*, 1998); and electroporation (Correnti and Pearce, 2004).

1.6.1 History and discovery of RNA interference

RNA interference was discovered in 1998 by the scientists Andrew Fire and Craig Mello. Prior to the RNAi discovery, two phenomena with similar effects to RNAi had been described. These phenomena are called “co-suppression” (Napoli *et al.*, 1990) and “quelling” (Romano and Macino, 1992) which had been described in the flower *Petunia hybrida* and in the fungus *Neurospora crassa*, respectively. The term “co-suppression” was coined by Napoli et al., 1990, to describe a gene silencing effect of the overexpression of an introduced chimeric gene, responsible for plant pigmentation. The aim of this experiment was to overexpress chalcone synthase

(CHS) in petunias with pigmented petals by introducing a chimeric chalcone synthase gene to the flowers. CHS is responsible for the plants' pigmentation, which was expected to increase after gene overexpression. Nonetheless, the overexpressed gene resulted in flowers with partially- or fully-white petals. This indicated that the introduction of the chimeric gene inhibited expression of both the introduced and the native CHS gene (Napoli *et al.*, 1990). Two years later, the term "quelling" was introduced to describe a gene silencing phenomenon in *N. crassa* which was similar to "co-suppression" in plants (Romano and Macino, 1992). Further experiments have been conducted in the plant *Petunia hybrida* to study at which stage the suppression of gene expression occurs in "co-suppression". It was shown that gene expression is suppressed post-transcriptionally, after the degradation of the mRNA (Van Blokland *et al.*, 1994). Nevertheless, the silencing mechanism remained unknown (Van Blokland *et al.*, 1994).

A cornerstone in the discovery of RNAi was an accidental finding of Guo and Kemphues in 1995. Specifically, they observed that the expression of a gene was silenced after injecting either anti-sense or sense RNA in *C. elegans* (Guo and Kemphues, 1995). Andrew Fire and Craig Mello took a step further and found that they could induce potent and highly specific gene silencing in *C. elegans* simply by injecting it with gene-specific dsRNA (Fire *et al.*, 1998). For this innovative discovery, Fire and Mello were awarded the Nobel Prize in Physiology or Medicine in 2006 ("The Nobel Prize in Physiology or Medicine 2006". Nobelprize.org. 16 May 2014 http://www.nobelprize.org/nobel_prizes/medicine/laureates/2006/).

1.6.2 RNAi pathway

Since the discovery of RNAi, a plethora of genetic and biochemical studies have been conducted in various organisms to decipher its pathway. Nowadays, there is a very good description of the RNAi pathway in *C. elegans*, which has been reviewed by many (Duxbury and Whang, 2004, Grishok, 2005, Hammond, 2005, Fischer, 2010). The pathway can be divided in three steps (Figure 1. 4). The first step includes uptake of the trigger dsRNA from intestinal lumen with the help of SID-2

(Systemic RNA interference Defective) protein (Winston *et al.*, 2007). Once dsRNA is in the cell, it is cleaved into the primary small inhibitory RNAs (siRNAs), which are ~22 nucleotides long with two nucleotide 3' end overhangs, by the RNase III enzyme Dicer (DCR-1) (Bernstein *et al.*, 2001, Knight and Bass, 2001). DCR-1 is part of a protein complex which additionally consists of the dsRNA binding RDE-4 (RNAi-Defective) protein (Tabara *et al.*, 2002); the Argonaute protein RDE-1 (Tabara *et al.*, 1999); and the DExH-box helicase DRH-1 (Tabara *et al.*, 2002). The second step is the amplification of the silencing signal by the production of secondary siRNAs, which are used for identification of the target mRNA. The key proteins for the production of secondary siRNA's are the RNA-dependent RNA-polymerases (RdRPs) (Sijen *et al.*, 2001). In *C. elegans,* the RdRPs which amplify the RNAi signal are: EGO-1 (Enhancer of Glp-One family member) in the germ cells (Smardon *et al.*, 2000) and RRF-1 (RNA-dependent RNA polymerase Family) and RDE-9 in the somatic cells (Sijen *et al.*, 2001). Studies have shown that the primary and the secondary siRNAs are two distinct classes of small RNAs, with the former aiding the production of the latter (Pak and Fire, 2007, Sijen *et al.*, 2007). In this respect, primary siRNAs guide RdRPs to the target mRNA, which is used as a template for the unprimed synthesis of the secondary siRNAs (Sijen *et al.*, 2007). The characteristics of the secondary siRNAs that distinguish them from primary siRNAs are that they have always anti-sense polarity (Sijen *et al.*, 2007); they carry 5' di- or triphosphates (Sijen *et al.*, 2007); and they are associated with the secondary Argonaute proteins (SAGOs) (Yigit *et al.*, 2006). The absence of Dicer's 5' monophosphate characteristic indicates that secondary siRNAs may be short RNA products from the beginning; or that they may be cleaved by a nuclease other than DCR-1; or even cleaved by DCR-1 and then modified in some way (Sijen *et al.*, 2007). Although the initiation of the secondary siRNAs has been proved, the final part of their production remains unknown.

The third and final step of the pathway is the nucleolytic degradation of the target mRNA by a protein complex called RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000). Biochemical studies revealed that this complex has a similar structure across species and particularly is comprised of an siRNA, an

Argonaute protein, the RNA binding protein VIG (Vasa Intronic Gne), the Tudor staphylococcal nuclease (Tudor-SN) and a Fragile X protein (Caudy *et al.*, 2003). Of all these, the Argonaute protein (Slicer) is believed to be responsible for cleavage of the target mRNA under the guidance of the siRNA (Hammond, 2005, Fischer, 2010). The Argonaute family members have both a PIWI and a PAZ domain which bind the 5' and 3' end of the siRNA, respectively (Hammond, 2005, Fischer, 2010). The PIWI domain contains an RNaseH fold (Song *et al.*, 2004), which is considered to cleave the target mRNA in the middle of the siRNA after the latter binds to the complementary region of the target mRNA by Watson-Crick base pairing (Hammond, 2005).

Since the discovery of RNAi, it was shown that the silencing effect of RNAi is not confined to the cells that are challenged with the trigger dsRNA, but it begins in these cells and spreads systemically (Fire *et al.*, 1998). This means that a gene can be silenced in a cell that does not contain the initial trigger dsRNA. There are many RSD (RNAi Spreading Defective) and SID proteins implicated in the systemic effect of RNAi. The transmembrane protein, which is encoded by the *sid-1/rsd-8/rde-7*gene, plays an important role in the passive cellular uptake of dsRNA (Winston *et al.*, 2002, Feinberg and Hunter, 2003). It is suggested that the dsRNA might enter adjoining cells via endocytosis (Tijsterman *et al.*, 2004). In addition, proteins such as RSD-2, RSD-3 and RSD-6 help the spread of the silencing signal from somatic to germ cells (Tijsterman *et al.*, 2004). The silencing effect of RNAi is temporary and its duration before the transcript of the target gene recovers to its normal levels depends on several *smg* (suppressor of morphological effects on genitalia) genes (Domeier *et al.*, 2000).

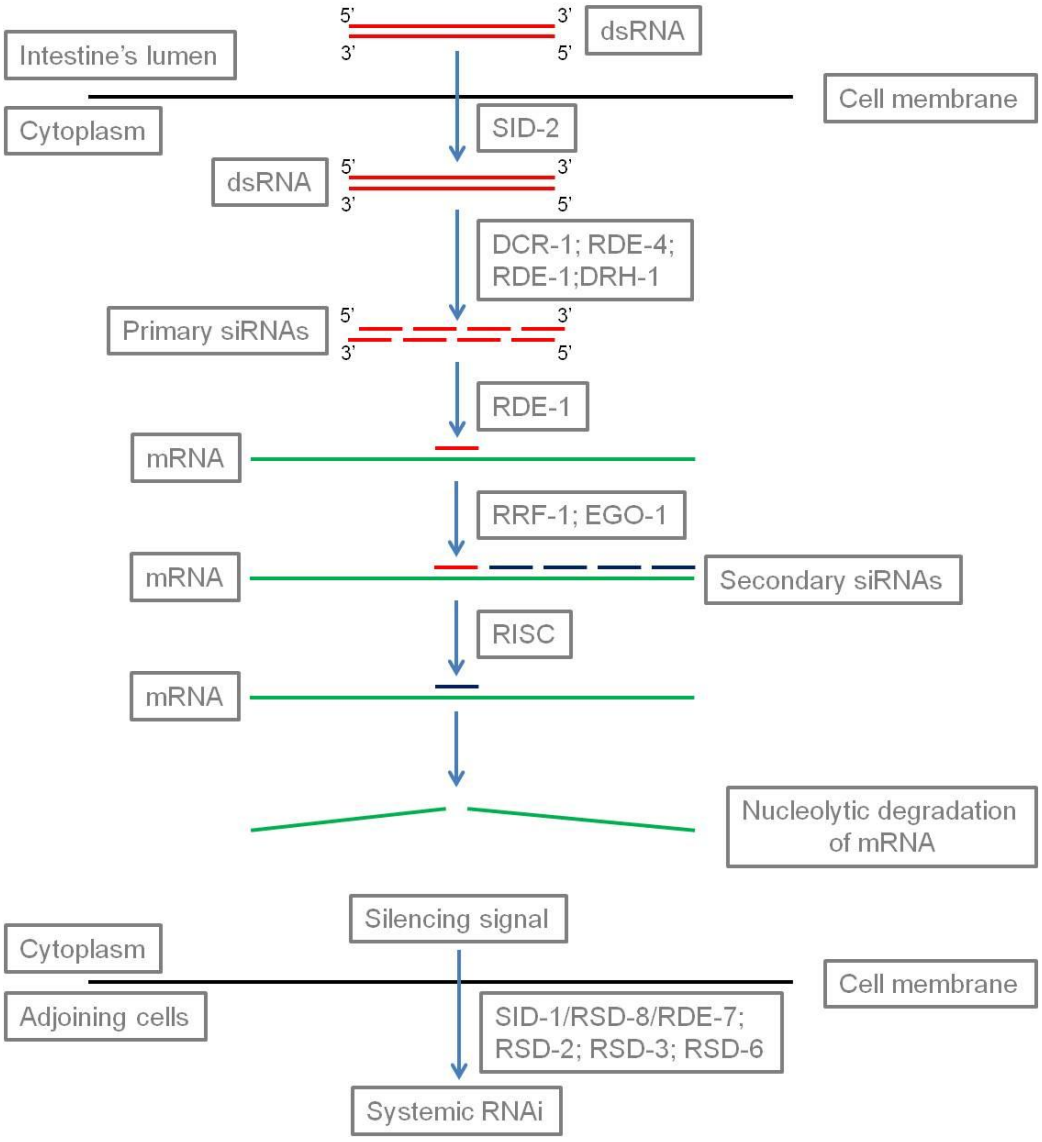


Figure 1.4. Proposed RNAi pathway

1.6.3 RNAi in parasitic nematodes

After the success of RNAi in *C. elegans*, many tried to apply this genetic tool to parasitic nematodes for the functional analysis of their genome. However, this application has proven difficult (Britton and Murray, 2006, Geldhof *et al.*, 2007). To date, RNAi has been applied with some success on a variety of parasitic nematodes which include, among others: *Nippostrongylus brasiliensis* (Hussein *et al.*, 2002), *T. colubriformis* (Issa *et al.*, 2005), *H. contortus* (Geldhof *et al.*, 2006b), and *O.*

ostertagi (Visser *et al.*, 2006). Nevertheless, the major problem confronting the routine use of RNAi as a tool to define gene function in parasitic nematodes is the great variability in results and the lack of consistency of knockdown, not only among different parasitic species, but also among different genes in the same species (Geldhof *et al.*, 2006b, Visser *et al.*, 2006). Although the reasons for this variability remain unknown, there are a number of suggestions to explain the lack of consistency of knock-down. In this respect, it has been suggested that the lack of consistency could be a consequence of inappropriate dsRNA delivery (Viney and Thompson, 2008); problematic culture conditions of the parasitic nematodes that do not allow them to continue development (Knox *et al.*, 2007); the presence of by-products during the synthesis of dsRNA, such as partially degraded dsRNA, anti-sense RNA or siRNAs, which could induce RNAi in high but not in low concentrations (Knox *et al.*, 2007); or the absence of essential genes required for successful RNAi (Viney and Thompson, 2008). Recently, it has been demonstrated that consistency of the silencing effect may depend on the expression site of the target-gene (Samarasinghe *et al.*, 2011). In particular, genes which are expressed in sites accessible from the environment (i.e. the intestine, excretory or amphid cells) appeared to be more susceptible to RNAi in *H. contortus* than genes expressed in other sites (Samarasinghe *et al.*, 2011). Furthermore, in the same study, silencing of those genes did not result in any phenotypic effect *in vitro*, but a reduction of worm survival and egg output was observed after challenging animals with RNAi-treated larvae (Samarasinghe *et al.*, 2011). The latter was a breakthrough observation, because it establishes *in vivo* RNAi as a valid method not only for identification and functional determination of essential parasite genes, but also as a screening tool for potential vaccine targets by infecting sheep with RNAi treated larvae and examining the outcome of the experimental infection (Samarasinghe *et al.*, 2011). However, a fundamental problem remains; why does RNAi work for some genes in an organism but not for others and why is there such variation amongst parasitic nematode species?

1.7 Aims of the project

The general aims of this project were, first, to use *C. elegans* to develop internal controls that would indicate activation of the RNAi pathway. This would help to define the status of the RNAi pathway genes after successful RNAi and, if successful, to apply these to investigating the pathway in *T. circumcincta*. The development of the internal controls to monitor pathway activity would be an aid to interpret inconsistent results here when applying RNAi in *T. circumcincta.* The next step was to focus on the development of an *in vitro* RNAi platform in *T. circumcincta* targeting ASP molecules, which are considered to be very important for the parasitism and as such, are valid vaccine candidates. The selection of candidate ASPs was enhanced by a detailed bioinformatics analysis and an analysis of stage-specificity of transcription using end-point PCR. Another aim was to investigate how ASPs relate to EMVs by confirming the presence or not of EMVs in *T. circumcincta*'s excretory/secretory products and by analyzing the protein content of these vesicles and their relationship to ASPs. Revealing novel ASPs as well as other molecules that are present in the EMVs would be of great benefit in the future selection of potential targets for pharmacological interventions against *T. circumcincta*.

Chapter 2: Marker genes that indicate the activation of the RNA interference pathway

2.1 Introduction

The discovery of RNA interference (RNAi) in 1998 was a breakthrough in biological sciences. This reverse genetic mechanism was initially described in *C. elegans* as a very robust method to silence the expression of genes (Fire *et al.*, 1998). The introduction of gene-specific double-stranded RNA (dsRNA) to *C. elegans*, after a series of events, leads to the gene knock-down. As mentioned before, the gene-specific dsRNA can be introduced to the worms in a variety of ways, such as: microinjection (Fire *et al.*, 1998); feeding worms *Escherichia coli* bacteria that express the gene-specific dsRNA (Timmons and Fire, 1998); soaking in a solution of gene-specific dsRNA (Tabara *et al.*, 1998); and electroporation (Correnti and Pearce, 2004). Soaking the worms in a solution of gene-specific dsRNA is the easiest of the delivery methods.

With the latter method, the dsRNA is ingested by the worms and is taken up by intestinal cells of the nematodes (Winston *et al.*, 2007). Once inside the cell, the dsRNA is cleaved into small inhibitory RNAs (siRNAs) by a protein complex which contains the enzyme Dicer-1 (DCR-1) (Bernstein *et al.*, 2001, Knight and Bass, 2001). Then, the silencing signal is amplified by the RNA-dependent RNA-polymerases (RdRPs) such as EGO-1 (Enhancer of Glp-One family member) with the production of more siRNAs (Smardon *et al.*, 2000, Sijen *et al.*, 2001). The siRNAs are used to identify the target mRNA by Watson-Crick base pairing, the mRNA is degraded (Hammond *et al.*, 2000) and this is how gene silencing is achieved. Finally, proteins like RSD-3 (RNAi Spreading Defective) aid in spreading the silencing signal throughout the organism (Tijsterman *et al.*, 2004). There are several reviews that have described the RNAi pathway (Duxbury and Whang, 2004, Grishok, 2005, Hammond, 2005, Fischer, 2010, Maule *et al.*, 2011) and a more detailed description of the pathway can be found in Section 1.6.2. of the General Introduction.

The highly specific gene-silencing caused by RNAi was extensively and successfully used for the functional analysis of the *C. elegans* genome (Maeda *et al.*, 2001, Kamath *et al.*, 2003). After the great success in *C. elegans*, the application of RNAi to define gene function was tested in a number of parasitic nematodes. To date, it has been applied to a variety of species which include, among others: *Trichostrongylus colubriformis* (Issa *et al.*, 2005), *Haemonchus contortus* (Geldhof *et al.*, 2006b), and *Ostertagia ostertagi* (Visser *et al.*, 2006). Nevertheless, the application of RNAi in parasitic nematodes has not proved as simple as in *C. elegans* (Britton and Murray, 2006, Geldhof *et al.*, 2007). The difficulty lies in the variability of the results and the lack of consistent silencing, not only among different species, but also among different genes within species (Geldhof *et al.*, 2006b, Visser *et al.*, 2006, Samarasinghe *et al.*, 2011).

Several suggestions exist in the literature to explain the inconsistency of these results including: potential inefficient delivery of the gene-specific dsRNA to the parasites; problematic culture conditions of the parasitic nematodes that do not allow them to continue their development; non-specific effects of the by-products during the synthesis of dsRNA; or the absence of the essential RNAi pathway genes in the parasitic nematode species (Knox *et al.*, 2007, Viney and Thompson, 2008). In addition, it has been demonstrated that consistency of the silencing effect may depend on the expression site of the gene (Samarasinghe *et al.*, 2011). In particular, genes expressed in sites accessible to the external environment (i.e. the intestine, excretory or amphid cells) appear to be more susceptible to RNAi in *H. contortus* than genes expressed in other sites (Samarasinghe *et al.*, 2011).

The application of RNAi in parasitic nematodes can be viewed as a ‘black box’ because there are several unanswered questions regarding the initiation of the RNAi pathway. In the unsuccessful cases, it is not known whether parasites’ cells took up the gene-specific dsRNA, and if so, whether the RNAi pathway was activated in response to this dsRNA. Hitherto, there have been no studies in the literature that have examined transcript levels of the RNAi pathway genes after exposure to exogenous gene-specific dsRNA. For example, are these genes constitutively transcribed or are they only “switched on” in response to exogenous dsRNA? The

answer to this question may shed some light on these issues, as these genes might be used as controls that are indicative of activation of the RNAi pathway. RNAi has evolved as a defence mechanism against the proliferation of foreign sequences, for example transposable elements or viruses, both of which replicate and produce dsRNA (Matzke *et al.*, 2001). Although there are reports in the literature regarding naturally and experimentally virus-infected *Caenorhabditis spp* nematodes (Felix *et al.*, 2011), there were no reports regarding viral infections in parasitic nematodes. As mentioned in the General Introduction, successful RNAi has been reported in both *C. elegans* (Fire *et al.*, 1998) and several parasitic nematodes (e.g. Britton and Murray, 2006, Geldhof *et al.*, 2007). This supports the rationale that RNAi has been evolved in these organisms and, as a result, exposure of the nematodes to dsRNA might result in altering the transcript levels of some of the RNAi pathway genes.

In this part of the project, three RNAi pathway genes were selected as potential markers of pathway operation in response to the presence of exogenous gene-specific dsRNA. The model organism that was used here was *C. elegans* due to the consistency and level of success of RNAi in this nematode observed previously (e.g. Geldhof *et al.*, 2006a). The three candidate marker genes which were chosen because of their central role in the RNAi pathway were *Ce-dcr-1, Ce-ego-1* and *Ce-rsd-3.* As mentioned before, the former gene encodes an RNase III enzyme that is required in the initial part of the pathway to cleave the dsRNA and produce the primary siRNAs (Bernstein *et al.*, 2001); Ce-EGO-1 uses the target mRNA as a template and produces more siRNAs in order to amplify the silencing signal (Maniar and Fire, 2011); and Ce-RSD-3 plays a role in the systemic spread of the silencing signal (Tijsterman *et al.*, 2004). Their potential as RNAi markers was examined in a series of RNAi experiments in *C. elegans*.

2.2 Materials and methods

2.2.1 *In vitro* culture of *Caenorhabditis elegans*

The adult stage of *C. elegans* (N2 strain; Brenner 1974) was used for the RNAi experiments. The nematodes were cultured *in vitro* as described previously

(Brenner, 1974). Briefly, they were maintained on NGM plates seeded with a uracil-requiring mutant of *E. coli* (OP50). This strain of bacteria is used as the bacterial lawn does not overgrow into a thick layer in the limited uracil medium and it does not obscure the worms (Brenner, 1974).

2.2.2 RNAi target gene selection, dsRNA preparation and establishing a successful RNAi protocol

Two target genes were selected, namely a cysteine protease encoding gene (*Ce-cpr-4*) and a superoxide dismutase encoding gene (*Ce-sod-4*), the first named being consistently susceptible (Geldhof *et al.*, 2006a) and the latter refractory to RNAi (Dave Knox, personal communication). The gene-specific dsRNAs (167 bp, *cpr-4*; 188 bp, *sod -4*) were prepared as described by Geldhof *et al.* (2006a). Firstly, it was confirmed that the areas chosen for dsRNA production did not contain the restriction sites for *Sac I* and *Sma I*, the primers were designed so that they had similar melting temperatures (Tm) and that the forward and the reverse primers had the *Sac I* (GAGCTC) and the *Sma I* (CCCGGG) restriction sites at the 5' and 3'prime ends, respectively (Table 2. 1). The target sequences were amplified from mRNA by reverse-transcriptase PCR (RT-PCR) using SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Life Technologies™) based on the manufacturer's instructions with the following cycling conditions: 50°C for 30 minutes; 94°C for 2 minutes; 40 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes; and finally a 7 minute extension at 72°C. Then, these amplicons were cloned into the L4440 plasmid vector after it was double digested with the restriction enzymes *Sac I* and *Sma I* and the plasmids were sequenced to confirm that the plasmids had the correct insert. Finally, each plasmid was linearized with the restriction enzymes *Sac I* and *Sma I* separately and the final dsRNA was produced from these templates by *in vitro* transcription using the T7 Ribomax™ Express RNAi System (Promega) following the manufacturer's guidelines. Briefly, in the first step of the protocol two complementary RNA strands were *in vitro* transcribed from the linearised plasmids. Subsequently, equal volumes of complementary RNA were mixed and incubated at 70°C for 10 minutes. Then, they

were slowly cooled to room temperature (for approximately 20 minutes). This allowed the annealing of the dsRNA. The preparation was then treated with both RNase A Solution and RQ1 RNase-Free DNase to remove any remaining single stranded RNA (ssRNA) and template DNA. The dsRNA was ethanol precipitated and the pellet containing the dsRNA was re-suspended in 60µl Nuclease-Free Water. 2µl of the dsRNA were electrophoresed in a 2% (w/v) agarose gel and visualized by staining with GelRed™ (Biotium, Cambridge Bioscience, Cambridge, UK) to examine whether the dsRNA had the correct size. The concentration of the dsRNA was measured using a nanodrop spectrophotometer (Nanodrop® ND-1000 UV–Vis Spectrophotometer) and the dsRNA was stored at -80ºC until needed for the RNAi experiments.

Initially, the RNAi soaking protocol described by Geldhof *et al.*, (2006a), was followed. Briefly, 15 adult *C. elegans* were incubated at room temperature for 24 hours in 15 µl of 1x PBS at a final concentration of 1 mg/ml dsRNA pre-mixed with 1 µl of lipofectamine (Life Technologies™). The experiment was conducted in triplicate. In each experiment, three soakings took place: one with *Ce-cpr-4* specific dsRNA, one with *Ce-sod-4* specific dsRNA and one with 1x phosphate buffer solution (PBS). After the 24 hour incubation period, total RNA was extracted from the worms as described in Section 2.2.3. The transcript levels of the target genes were estimated by reverse-transcriptase PCR (RT-PCR) with pairs of specific primers (Table 2. 2) as described in Section 2.2.3.

A time-course experiment was conducted to determine the best duration for the soaking period. The protocol was adapted by increasing the number of the soaking worms to 50 with the simultaneous increase of the volumes of PBS and lipofectamine, but keeping the final concentration of dsRNA the same. Hence, 50 adult *C. elegans* were incubated at room temperature in 45 µl of 1x PBS at a final concentration of 1 mg/ml dsRNA pre-mixed with 3 µl of lipofectamine for 15 minutes, 1 hour, 24 hours and 48 hours. After soaking, total RNA was extracted from the worms (see Section 2.2.3.). Again, this experiment was conducted in triplicate.

A separate experiment was conducted in duplicate to test whether manual or chemical lysis of the worms is better for the extraction of total RNA. Two RNA preparations from 15 adult *C. elegans* were performed with a hand-held glass Teflon® homogenizer and another two with lysis buffer (0.5% SDS, 5% β-mercaptoethanol, 10mM EDTA, 10mM TRIS-HCL pH 7.5, 0.5 mg/ml proteinase K). The only difference between the two protocols was the homogenization step. For the manual lysis of the cells, the worms were transferred in the hand-held glass Teflon homogenizer which contained TRIzol® reagent (Life Technologies™). The homogenization of the worms took place in the reagent and then the manufacturer's instructions were followed. For the chemical lysis of the cells, the lysis buffer was added to the tube with the worms, frozen to -80°C, incubated to 55°C and then the TRIzol® reagent (Life Technologies™) was added and the manufacturer's instructions were followed. The RNA was treated with RQ1 RNase-Free DNase (Promega) and the transcript levels of the *Ce-cpr-4* and *Ce-sod-4* genes were examined by SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Life Technologies™) as described in Section 2.2.3.

Table 2.1. Accession numbers, primer sequences and product sizes of the target genes for the double-stranded RNA production. All the sequences are orientated from 5' to 3' end.

Gene	Accession number	Forward primer	Reverse primer	Product size
cpr-4	NM_072281	GAGCTCATCG TTTCTGCATCG CCTCC	CCCGGGCTG AGCCTCGTA AGATCCTCC	167 bp
sod-4	AB 190513	GAGCTCAAGT GATTCGTGCA CGTGCC	CCCGGGTTA GACAACCAT TTCCAGTATC TCC	188 bp

2.2.3 Development of the RNAi pathway marker genes

As noted above, *Ce-cpr-4* and *Ce-sod-4* were targeted. The experiments for the development of the marker genes were conducted in quadruplicate. In each experiment three soakings took place: one with *Ce-cpr-4* specific dsRNA, one with *Ce-sod-4* specific dsRNA and one with 1xPBS. Based on the results of the time-course experiment (Section 2.3.1.), the following soaking protocol was selected: 50 adult *C. elegans* were incubated for 1 hour at room temperature in 45 µl of 1x PBS containing 1 mg/ml dsRNA pre-mixed with 3 µl of lipofectamine. After the end of the incubation period, total RNA was extracted from the worms using the hand-held glass Teflon homogenizer and TRIzol® reagent (Life Technologies™) based on the instructions of the manufacturer. The concentration of the total RNA was measured using a nanodrop spectrophotometer (Nanodrop® ND-1000 UV–Vis Spectrophotometer). Finally, the total RNA was treated with RQ1 RNase-Free DNase (Promega) to degrade any genomic DNA contamination following the manufacturer's protocol.

Approximately 100ng of DNase treated total RNA were used as a template in a reverse-transcriptase PCR (RT-PCR) employing primers specific for *Ce-cpr-4*, *Ce-sod-4*, *Ce-dcr-1*, *Ce-rsd-3* and *Ce-ego-1* (Table 2. 2). Equal loading and integrity of each total RNA preparation were verified by amplifying a fragment of the *C. elegans pmp-3* (Peroxisomal Membrane Protein) gene (Table 2. 2). SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Life Technologies™) was used based on the manufacturer's instructions with the following cycling conditions: 50°C for 30 minutes; 94°C for 2 minutes; 40 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes; and a 7 minute extension at 72°C. Amplification products were separated on 2% (w/v) agarose gels and visualized by staining with GelRed™ (Biotium, Cambridge Bioscience, Cambridge, UK). The DNA products were extracted from the agarose gel using a QIAquick gel extraction kit (Qiagen), cloned into the pGEM®-T easy vector (Promega) according to the manufacturers' instructions and sent for sequencing to Eurofins (http://www.eurofinsgenomics.eu/) to confirm that the correct genes were amplified.

Finally, a bioinformatics search was made to confirm PCR product size if genomic DNA was amplified with the same primers.

The same amount of total RNA as in end-point PCR was used as a template in the reverse-transcriptase quantitative PCR (qRT-PCR). Gene specific primers and hydrolysis probes were designed using Primer Express 3.0 software (Applied Biosystems) for *Ce-dcr-1, Ce-rsd-3, Ce-ego-1, Ce-pmp-3* and *Ce-y45f10d.4* (Table 2. 3). Both primers and probes were HPLC (High-Performance Liquid Chromatography) purified and purchased from Eurofins MWG operon (http://www.eurofinsdna.com). The hydrolysis probes were labelled with the fluorescent dye FAM (Fluorescein amidite) at the 5' end and the non-fluorescent quencher dye BHQ1 (black hole quencher) at the 3' end. The qRT-PCR was performed using SuperScript® III Platinum®One-Step qRT-PCR kit (Life Technologies™) and the ABI 7500 system (Applied Biosystems) based on the manufacturer's instructions with the following cycling conditions: 50°C for 15 minutes; 95°C for 2 minutes; and 40 cycles of 95°C for 15 seconds, 57°C for 30 seconds and 60°C for 32 seconds. Each reaction in qRT-PCR was run in duplicate and standard curves were generated using five, 4-fold dilutions of the total RNA template to determine the efficiency of the primers. Negative (no template) controls were also included. Finally, *Ce-pmp-3* and *Ce-y45f10d.4* were chosen as house-keeping genes based on previous studies, due to their stable expression levels (Hoogewijs *et al.*, 2008).

The relative transcript levels of the candidate marker genes in the worms soaked in *Ce-cpr-4* and *Ce-sod-4* dsRNA were calculated as a fold change compared to untreated worms (soaked in 1xPBS) and normalized against both control genes (*Ce-pmp-3* and *Ce-y45f10d.4*) using the formula:

fold change = $2^{-\Delta\Delta Ct}$; where, $\Delta\Delta C_t = \Delta C_{t\ treated} - \Delta C_{t\ untreated}$; $\Delta C_{t\ treated} = C_{t\ marker\ gene\ treated} - C_{t\ housekeeping\ gene\ treated}$; $\Delta C_{t\ untreated} = C_{t\ marker\ gene\ untreated} - C_{t\ housekeeping\ gene\ untreated}$.

Minitab® 15.1.0.0 was used for the statistical analysis of the qRT-PCR results. Normality of the data was tested with Anderson-Darling Normality test. A

two sample t-test was used to test whether there was a difference in the relative transcript levels of the pathway genes between worms with activated and inactivated RNAi pathways. Significant results were considered as having a p value < 0.05.

Table 2.2. Accession numbers, primer sequences and product sizes of the genes of interest for the end-point RT-PCR in *C. elegans*. All the sequences are orientated form 5' to 3' end.

Gene	Accession number	Forward primer	Reverse primer	Product size
cpr-4	NM_072281	ATTTGCTCTATCT TGCTATTTGC	CTGAGCCTCGT AAGATCCTCC	563 bp
sod-4	AB190513	AAGTGATTCGTG CACGTGCC	TTAGACAACCA TTTCCAGTATCT CC	189 bp
dcr-1	NM_066360.2	AGAGCTGATTTA CAATGTTTTAAC C	GATAGCTTGTT GTTCAACGAGG	228 bp
rsd-3	NM_077572	AATCACAAAAAA CGAGTATGTCC	AATTTGCCTTTT GTCGTTCAGC	467 bp
ego-1	AF159143	ATCTACCTCAAA ACGATTTTAGCC	TTGGAATGGCA TAAGATGATCC	355 bp
pmp-3	NM_074219.2	GTTCCCGTGTTC ATCACTCAT	ACACCGTCGAG AAGCTGTAGA	115 bp

Table 2.3. Accession numbers, primer and probe sequences and product size of the genes of interest for the qRT-PCR in *C. elegans*. All the sequences are orientated from 5' to 3' end.

Gene	Accession number	Forward primer	Reverse primer	Probe	Product size
dcr-1	NM_066360.2	AGCCCGTGTTGGTAATGATGA	CAGCGATTGATTTATCCGAAATATG	TCCACTTCCTTACAACTTGCTCACCCAACA	83 bp
rsd-3	NM_077572	TGCCGGACTCGACCAGTT	TTGGTTAAGTGTTTTTGCTTTTGG	CGTTGGGATCCACAAACTCGAATGC	79 bp
ego-1	AF159143	GATCAATCTGTTGGGCTCAATG	TGTCCGCGAATTCAATGGA	CTACCGCTTACTTTCAAGACTTCGCATCCG	85 bp
pmp-3	NM_074219.2	TTCTTCCTGGCTCAAAAACCA	GCTTTGACTGGATATACGATTTGTTG	TTCCCATCTGGAAACACCACTCTGCG	77 bp
y45f10d.4	NM_070257.2	CGGATGTGGAAGTGCAATTG	CGACGCGTAATCGATAGTTTTTC	CATCTTCCCTGGCAACCGAATGGA	76 bp

2.3 Results

2.3.1 Establishing a successful RNAi protocol

The sequencing results for the L4440 plasmids confirmed that the plasmids contained the correct insert sequence for the target genes (*Ce-cpr-4* and *Ce-sod-4*). Soaking 15 adult *C. elegans* in *Ce-cpr-4* specific dsRNA for 24 hours resulted in a loss of transcript for *Ce-cpr-4* gene, evident as a reduction in the intensity of the signal (Figure 2. 1, Lane 2) compared to the control sample (Figure 2. 1, Lane 4). In contrast, soaking in *Ce-sod-4* specific dsRNA for 24 hours had no effect on *Ce-sod-4* transcript compared to the control sample (Figure 2. 1). Soaking in *Ce-sod-4* specific dsRNA had no effect on *Ce-cpr-4* transcript levels, and *vice versa* (Figure 2. 1). These results were reproduced in triplicate.

Soaking 50 adult *C. elegans* in *Ce-cpr-4* and *Ce-sod-4* dsRNA for 15 minutes, 1 hour, 24 hours and 48 hours led to a loss of transcript for all the incubation periods only for *Ce-cpr-4* (Figures 2. 2 and 2. 3), with the most robust being after 1 hour incubation period (Figure 2. 2). These results were identical in the three biological replicates.

After homogenizing 15 adult *C. elegans* in two different ways, i.e. manually and chemically, RT-PCR was performed to examine the transcript levels of *Ce-cpr-4* and *Ce-sod-4* genes. The results were the same in the two biological replicates and showed that manual was better than chemical homogenization (Figure 2. 4).

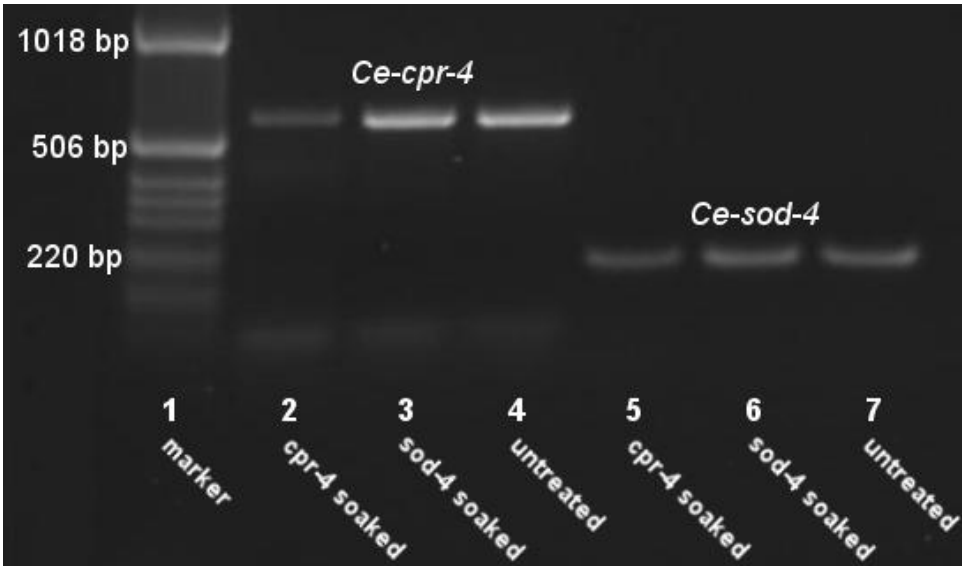


Figure 2.1. RT-PCR detection of transcript levels of *Ce-cpr-4* and *Ce-sod-4* after soaking 15 adult *C. elegans* for 24 hours in *Ce-cpr-4* dsRNA, *Ce-sod-4* dsRNA and 1xPBS. Lanes 2, 3 and 4 represent the *Ce-cpr-4* transcript levels of worms soaked in *Ce-cpr-4* specific dsRNA, *Ce-sod-4* specific dsRNA and 1xPBS, respectively. Likewise, lanes 5, 6 and 7 represent the *Ce-sod-4* transcript levels of worms soaked in *Ce-cpr-4* specific dsRNA, *Ce-sod-4* specific dsRNA and 1xPBS, respectively. Soaking worms in *Ce-cpr-4* dsRNA resulted in reduced transcript abundance of the target gene (lane 2 compared to lane 4), whilst soaking in *Ce-sod-4* dsRNA did not result in any effect on the target gene (lane 6 compared to lane 7). Soaking in *Ce-sod-4* dsRNA had no effect on the non-target gene *Ce-cpr-4* (lane 3 compared to lane 2) and *vice versa* (lane 5 compared to lane 6). The first lane shows a 1 kb DNA marker.

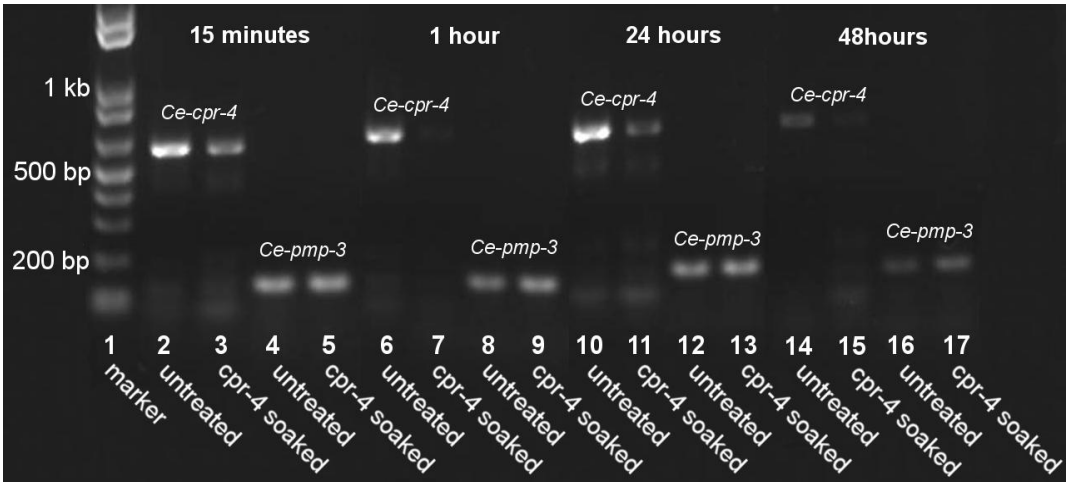


Figure 2.2. RT-PCR detection of transcript levels of *Ce-cpr-4* and *Ce-pmp-3* following soaking of 50 adult *C. elegans* in *Ce-cpr-4* specific dsRNA for 15 minutes (lanes 2 to 5), 1 hour (lanes 6 to 9), 24 hours (lanes 10 to 13) and 48 hours (lanes 14 to 17). Transcripts are shown for *Ce-cpr-4* (lanes 2-3, 6-7, 10-11, 14-15) and *Ce-pmp-3* (lanes 4-5, 8-9, 12-13, 16-17). The even numbered lanes represent the transcripts of control worms (soaked in 1xPBS only) and the odd numbered lanes, the dsRNA-treated worms. *Ce-pmp-3* was used as a transcript control. Knock-down of *Ce-cpr-4* was observed in all the incubation periods (lanes 3, 7, 11 and 15 compared to lanes 2, 6, 10 and 14, respectively) with the most robust effect after 1 hour of incubation in dsRNA. In each case, transcript levels of *Ce-pmp-3* were unaffected. The first lane shows a 1 kb DNA marker.

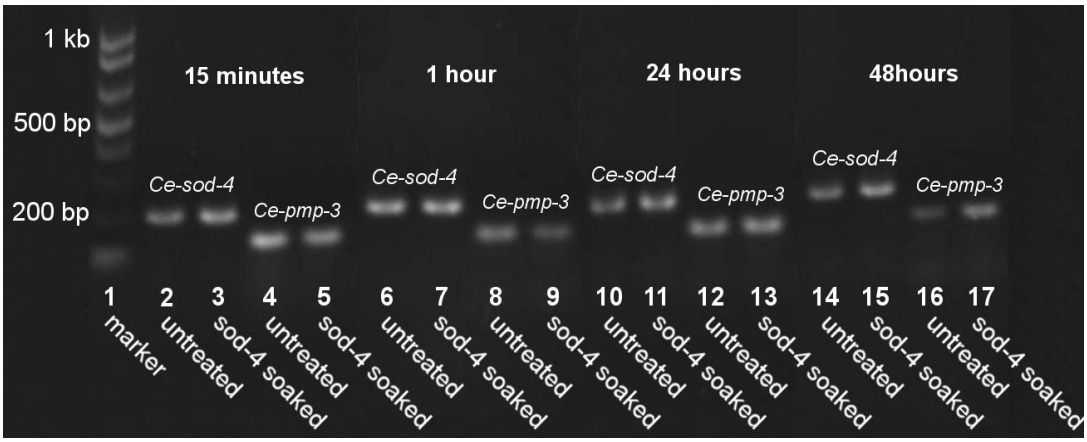


Figure 2.3. RT-PCR detection of transcript levels of *Ce-sod-4* and *Ce-pmp-3* following soaking of 50 adult *C. elegans* in *Ce-sod-4* specific dsRNA for 15 minutes (lanes 2 to 5), 1 hour (lanes 6 to 9), 24 h (lanes 10 to 13) and 48 hours (lanes 14 to 17). Transcript levels are shown for *Ce-sod-4* (lanes 2-3, 6-7, 10-11, 14-15) and *Ce-pmp-3* (lanes 4-5, 8-9, 12-13, 16-17). The even numbered lanes represent the transcripts of control worms (soaked in 1xPBS) and the odd numbered

lanes, the dsRNA treated worms. *Ce-pmp-3* was used as a transcript control. Knock-down of *Ce-sod-4* was not observed in any of the incubation periods (lanes 3, 7, 11 and 15 compared to lanes 2, 6, 10 and 14, respectively). The first lane shows a 1 kb DNA marker.

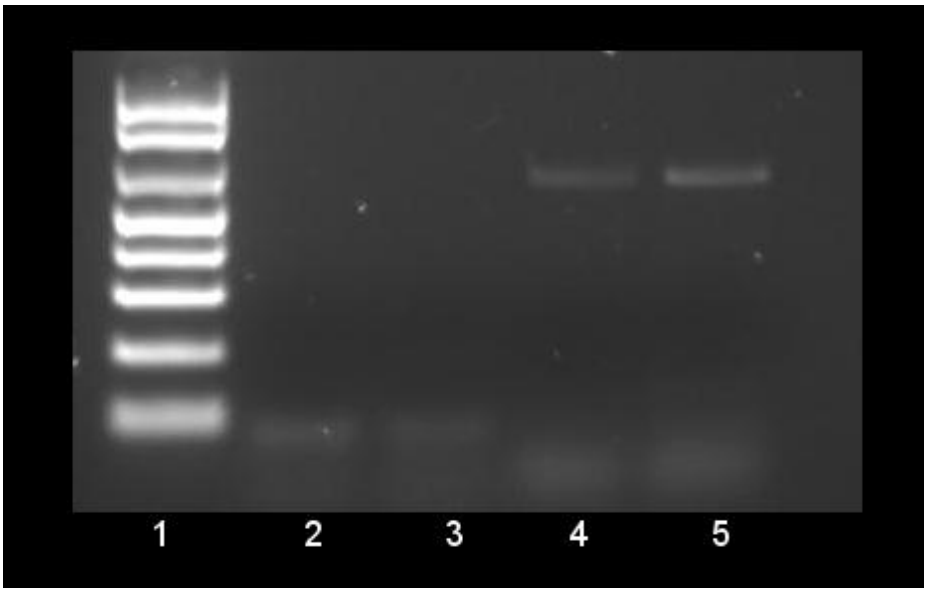


Figure 2.4. RT-PCR detection of transcript levels of *Ce-cpr-4* after chemical and manual RNA extraction. Transcript levels of *Ce-cpr-4* after chemical lysis and after manual lysis of the worms can be seen in lanes 2-3 and 4-5, respectively. The data indicate that the manual method was better than the chemical RNA extraction. The first lane shows a 1 kb DNA marker.

2.3.2 Assessment of the potential RNAi pathway marker genes

The end point RT-PCR experiments showed a marked reduction in the *Ce-cpr-4* transcript of the worms soaked in *Ce-cpr-4* specific dsRNA, but no change in the *Ce-sod-4* transcript of the worms soaked in *Ce-sod-4* specific dsRNA (Figure 2. 5). The candidate marker genes (*Ce-dcr-1*,*Ce-ego-1* and *Ce-rsd-3*), showed a stable transcription pattern regardless of the treatment the worms received, since the intensity of the bands was the same when the RNAi pathway was active (exposure to *Ce- cpr-4* dsRNA, Figure 2. 6) and when it was not (exposure to *Ce-sod-4* dsRNA, Figure 2. 6). These results were reproduced in quadruplicate. The sequencing results

for the amplification products showed that the correct genes were amplified at the endpoint RT-PCR, i.e. *Ce-cpr-4*, *Ce-sod-4*, *Ce-dcr-1*, *Ce-ego-1*, *Ce-rsd-3* and *Ce-pmp-3*. Moreover, the bioinformatics search showed that the amplification products would be larger if the samples were contaminated with genomic DNA. It was found that an intron was present between the primer binding sites in the genomic DNA. In particular, in the RNAi target genes *Ce-cpr-4* and *Ce-sod-4*, there were introns with lengths 49 bp and 526 bp, respectively. The lengths of the introns in the RNAi pathway genes and the house-keeping gene were: 130 bp in *Ce-dcr-1*, 86 bp in *Ce-ego-1*, 1511 bp in *Ce-rsd-3* and 2389 bp in *Ce-pmp-3*.

The results of the qRT-PCR experiments support the results of the end-point RT-PCR study (Table 2. 4 and Figure 2. 7). The Anderson Darling Normality test showed that the data were normally distributed ($p>0.05$) and the two sample t-test showed no significant difference between activation or not of the RNAi pathway for any of the pathway genes ($p>0.05$).

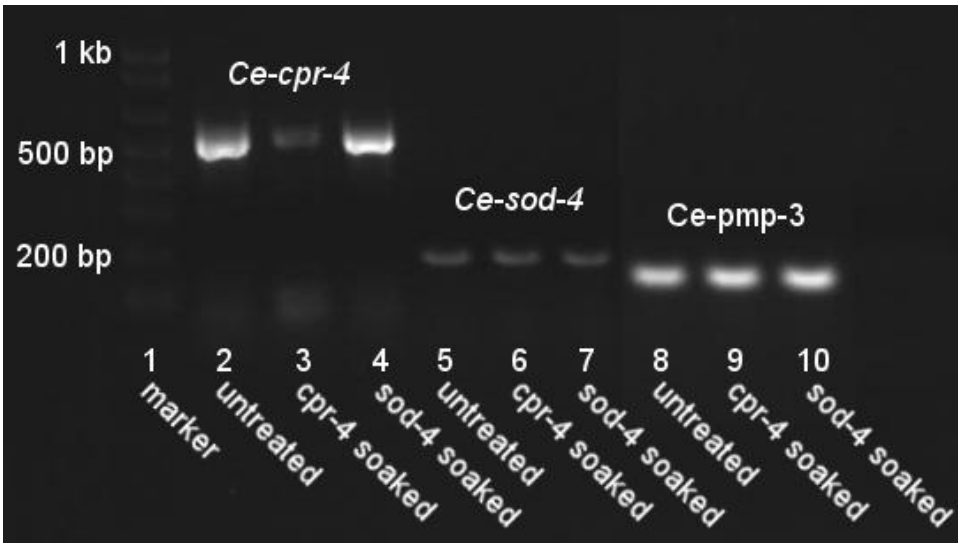


Figure 2.5. RT-PCR detection of transcript levels of *Ce-cpr-4, Ce-sod-4* and *Ce-pmp-3* following soaking of 50 adult *C. elegans* for 1 hour in *Ce-cpr-4, Ce-sod-4* specific dsRNA and 1xPBS. Lanes 2, 5, 8 correspond to the worms soaked in 1xPBS. Lanes 3, 6, 9 to the worms soaked in *Ce-cpr-4* specific dsRNA and lanes 4, 7, 10 to the worms soaked in *Ce-sod-4* specific dsRNA. Lanes 2-4, 5-7 and 8-10 represent the transcript levels of *Ce-cpr-4, Ce-sod-4* and *Ce-pmp-3,* respectively. *Ce-*

pmp-3 **was used as a housekeeping gene. Soaking worms in** ***Ce-cpr-4*** **dsRNA resulted to a specific down-regulation of the target gene (lane 3 compared to lanes 2, 4, 8 and 9). In contrast, soaking in** ***Ce-sod-4*** **dsRNA did not result to a down-regulation of the target gene (lane 7 compared to lanes 5, 6, 8 and 10). The first lane shows a 1 kb DNA molecular weight marker.**

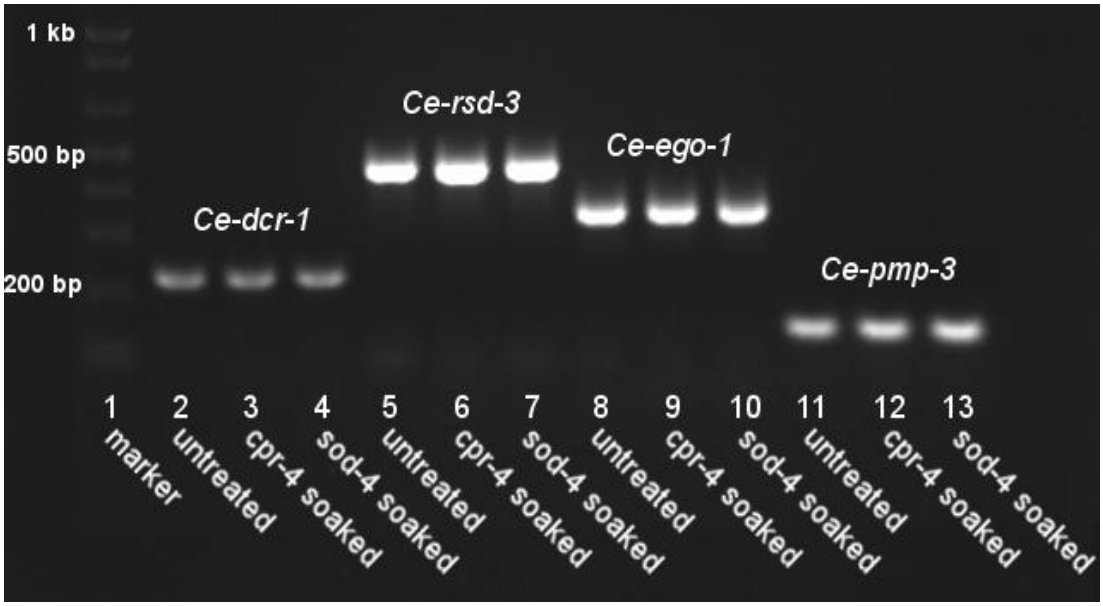


Figure 2.6. RT-PCR detection of transcript levels of ***Ce-dcr-1, Ce-rsd-3, Ce-ego-1 and Ce-pmp-3*** **following soaking of 50 adult** ***C. elegans*** **for 1 hour in** ***Ce-cpr-4, Ce-sod-4*** **specific dsRNA and 1xPBS. Lanes 2, 5, 8, 11 correspond to the worms soaked in 1xPBS. Lanes 3, 6, 9, 12 to the worms soaked in** ***Ce-cpr-4*** **specific dsRNA and lanes 4, 7, 10, 13 to the worms soaked in** ***Ce-sod-4*** **specific dsRNA. Lanes 2-4, 5-7, 8-10 and 11-13 represent the transcript levels of** ***Ce-dcr-1, Ce-rsd-3, Ce-ego-1 and Ce-pmp-3,*** **respectively.** ***Ce-pmp-3*** **was used as a housekeeping gene. The transcript levels of the pathway genes were the same regardless the activation or not of the RNAi pathway since the intensity of the bands is similar between the treatments (lane 3 compared to lanes 2 and 4; lane 6 compared to lanes 5 and 7; and lane 9 compared to lane 8 and 10). The first lane shows a 1 kb DNA molecular weight marker.**

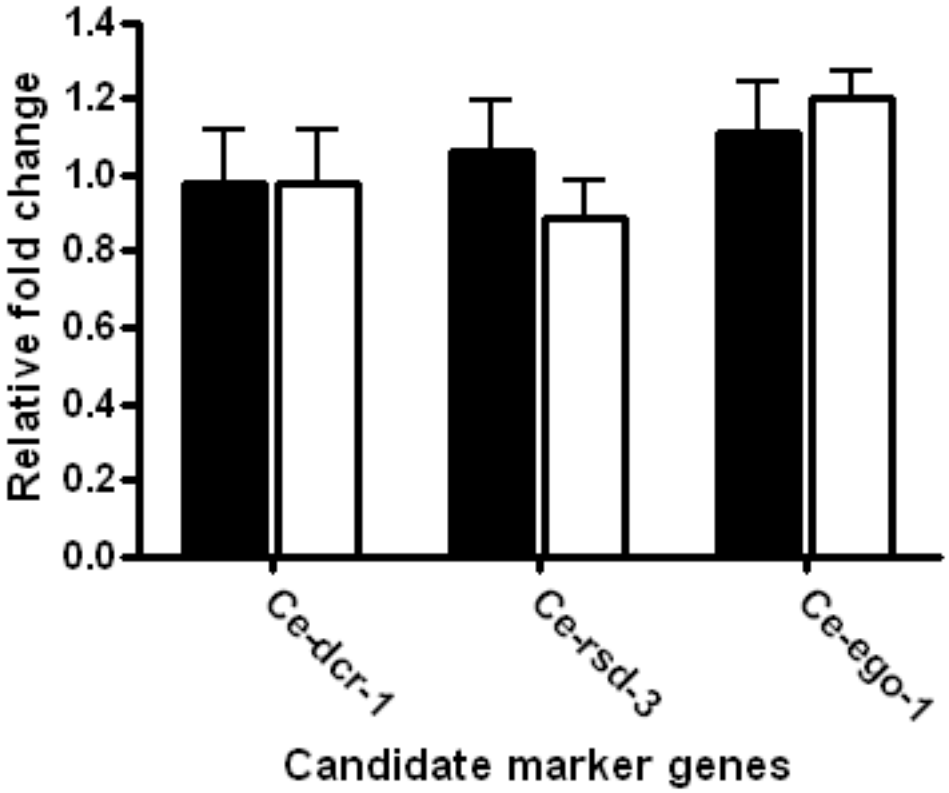


Figure 2.7. Fold-change of the three candidate RNAi pathway genes in the two treatments (activated and inactivated RNAi pathway) compared to untreated worms (soaked in 1xPBS) and normalized against both control genes (*Ce-pmp-3* and *Ce-y45f10d.4*). The black and white columns represent the mean fold-change of the transcripts in worms soaked in *Ce-cpr-4* dsRNA (activated pathway) and *Ce-sod-4* dsRNA (inactivated pathway), respectively, following four biological replicates. The error bars represent the standard error of the mean fold change of the candidate RNAi pathway genes following 4 biological replicates. No significant difference was found between the two treatments in any of the pathway genes.

Table 2.4. Individual Ct values of the three candidate RNAi pathway genes and the two control genes in four biological experiments with each one including three different treatments, ie soaked in 1xPBS (untreated); soaked in *Ce-cpr-4* specific dsRNA (activated RNAi pathway); and soaked in *Ce-sod-4* specific dsRNA (inactivated RNAi pathway).

		Ct values				
	treatment	**Ce-dcr-1**	**Ce-rsd-3**	**Ce-ego-1**	**Ce-pmp-3**	**Ce-y45f10d**
exp 1	1xPBS	20.68	20.89	19.985	21.33	19.085
	Ce-cpr-4 dsRNA	21.29	21.135	20.27	22.3	19.475
	Ce-sod-4 dsRNA	20.485	20.77	19.665	21.37	18.93
exp 2	1xPBS	27.64	28.05	27.055	27.37	26.205
	Ce-cpr-4 dsRNA	26.925	27.45	26.55	26.78	25.62
	Ce-sod-4 dsRNA	24.735	24.805	23.8	24.12	23.16
exp 3	1xPBS	23.635	24.075	23.3	23.595	22.94
	Ce-cpr-4 dsRNA	23.975	24.065	23.06	23.255	22.275
	Ce-sod-4 dsRNA	23.555	23.865	22.41	23.065	22.105
exp 4	1xPBS	27.485	27.265	27.345	26.695	25.055
	Ce-cpr-4 dsRNA	24.51	24.33	24.21	23.81	22.535

		Ct values				
	treatment	**Ce-dcr-1**	**Ce-rsd-3**	**Ce-ego-1**	**Ce-pmp-3**	**Ce-y45f10d**
	Ce-sod-4 dsRNA	26.54	27.195	26.305	25.935	24.68

2.4 Discussion

Work in this chapter showed that the silencing effect of RNAi could be observed after soaking adult *C. elegans* in a dsRNA solution for as little as 1 hour, which to my knowledge has not been reported previously. In addition, measurement of transcription levels of the *Ce-dcr-1, Ce-ego-1* and *Ce-rsd-3* genes showed that the transcript levels of those genes were similar regardless of whether degradation of target gene transcript had occurred or not. As a result, those genes could not be used as marker genes that would indicate the activation of the RNAi pathway.

The selection of the two target genes was based on their susceptibility to RNAi. The *Ce-cpr-4* gene was proven to be consistently susceptible to RNAi in previous studies (Geldhof *et al.*, 2006a), whilst *Ce-sod-4* was shown to be consistently refractory to RNAi, in our hands (Dave Knox, personal communication). The end-point RT-PCR results for the target genes in *C. elegans* showing successful silencing for *Ce-cpr-4* but not for *Ce-sod-4*, were consistent with previous observations (Geldhof *et al.*, 2006a). Moreover, specificity of the silencing effect was illustrated by the fact that soaking worms in *Ce-sod-4* specific dsRNA had no effect on *Ce-cpr-4* transcript levels, and *vice versa*. The lack of *Ce-sod-4* transcript decrease, here, was translated as a failure of the *Ce-sod-4* specific dsRNA to activate the RNAi pathway. Nevertheless, a small possibility could be that it was harder to detect the silencing effect than the *Ce-cpr-4* due to higher expression levels or higher stability of the *Ce-sod-4* mRNA. The comparison of the two lysis methods, i.e. chemical and manual, showed that the manual lysis of the worms was better and this method was used for the remainder of the project.

Once the RNAi methodology was established and the results successfully reproduced, the soaking protocol was adapted and a time course experiment performed. The number of the worms was increased to 50 adult *C. elegans* which were soaked for 15 minutes, 1, 24 and 48 hours. From this experiment it was observed that the transcript abundance of *Ce-cpr-4* was reduced after 15 minutes soaking with a successful knockdown effect after soaking the worms for 1 and 24 hours. After 48 hours of soaking, a number of worms died, potentially due to over-crowding effects and the data shown for this time point in Figures 2. 2 and 2. 3 were viewed as variable and unreliable. The fact that RNAi is successful after only 1 hour of soaking can be explained considering that RNAi is the cellular anti-viral defence mechanism (Vance and Vaucheret, 2001) and as a result, it should be activated shortly after the exposure to "intruding" dsRNA. After this experiment, the soaking period of 1 hour was adopted for further experiments to examine whether the selected RNAi pathway genes (*Ce-dcr-1*, *Ce-ego-1* and *Ce-rsd-3*) could be used as internal controls for the activation of the RNAi pathway. These pathway genes were chosen as they are highly conserved amongst parasitic nematodes (Dalzell *et al.*, 2011) and they allow analysis of components of different parts of the RNAi pathway. The rationale behind the choice of the soaking period was that any significant changes in the transcript levels of the pathway genes would be easier to observe shortly after the RNAi pathway activation.

The results of the end-point RT-PCR for the candidate marker genes showed no significant difference in transcript levels among worms with an activated RNAi pathway (i.e. soaked in *Ce-cpr-4* specific dsRNA); an inactivated RNAi pathway (i.e. soaked in *Ce-sod-4* specific dsRNA); and the untreated worms (i.e. soaked in 1xPBS). Despite the fact that end-point RT-PCR is a sensitive and specific technique, there cannot be drawn any definitive conclusions since this method is not an accurate quantification tool (Raeymaekers, 1995). In particular, the analysis of the data here required the comparison of the band intensity after 40 cycles of PCR, at which point the reactions might have been saturated and any differences in the initial transcript levels might not have been obvious in the agarose gel. As a result, the analyses were repeated using qRT-PCR.

Quantitative PCR eliminates the disadvantage of analyzing the data when the reactions have reached the plateau phase since the data from each individual cycle is shown and the exponential amplification phase is used for the subsequent analysis of the results (Gibson *et al.*, 1996, Heid *et al.*, 1996). This allows the accurate and simultaneous analysis of several genes (Gibson *et al.*, 1996, Heid *et al.*, 1996). In order to have reliable results for the transcription pattern of the candidate pathway genes, a set of housekeeping genes had to be chosen for accurate normalization of gene-expression levels (Vandesompele *et al.*, 2002). The choice of the housekeeping genes that were used in this study [*Ce-pmp-3* (Peroxisomal Membrane Protein) and *Ce-y45f10d.4*] was based on previous studies which showed their stability of expression (Hoogewijs *et al.*, 2008). The transcript levels of the candidate marker genes was shown as a fold-increase or -decrease by using the formula: fold increase= $2^{-\Delta\Delta Ct}$. [where, $\Delta\Delta C_t = \Delta C_{t\ treated} - \Delta C_{t\ untreated}$; $\Delta C_{t\ treated} = C_{t\ marker\ gene\ treated} - C_{t\ housekeeping\ gene\ treated}$; $\Delta C_{t\ untreated} = C_{t\ marker\ gene\ untreated} - C_{t\ housekeeping\ gene\ untreated}$]. The number "2" in the formula indicates the efficiency of the primers as, in theory, the amount of the product doubles after every cycle. Based on the melting curves, the efficiency of the primers used here was between 95 and 99%, which corresponds to an amplification rate between 1.95-1.99. The results of the qRT-PCR confirmed the end-point RT-PCR data showing that the transcript levels of the candidate marker genes were not significantly different between the *C. elegans* samples with activated and inactivated RNAi pathways ($p>0.05$). As a result, the chosen candidate pathway genes do not qualify as markers for activation of the RNAi pathway in response to exogenous dsRNA.

The stable transcript levels of the chosen RNAi pathway genes might be due to the presence of other molecules and different small RNA pathways that are used to regulate gene expression in *C. elegans* (Fischer, 2010). Particularly, apart from the siRNAs that are produced by the cleavage of exogenous dsRNA (Fire *et al.*, 1998), there are also endogenous siRNAs that originate from endogenous loci (Duchaine *et al.*, 2006, Lee *et al.*, 2006, Guang *et al.*, 2008). These endogenous siRNAs potentially regulate the transcript levels of genes by using part of the RNAi pathway including *Ce-dcr-1*, *Ce-ego-1* and *Ce-rsd-3* (Duchaine *et al.*, 2006, Lee *et al.*, 2006,

Guang *et al.*, 2008). Other molecules that might use part of the RNAi pathway are the microRNAs (miRNAs). The latter are involved in the inhibition of translation and/or the stability of target mRNAs (Lee *et al.*, 1993, Pasquinelli *et al.*, 2000, Reinhart *et al.*, 2000) and it was found that *Ce-dcr-1* is required for their biogenesis (Bernstein *et al.*, 2001, Hutvagner *et al.*, 2001, Ketting *et al.*, 2001). Since the aforementioned small RNA pathways use the proteins encoded by *Ce-dcr-1*, *Ce-ego-1* and *Ce-rsd-3*, it is rational to assume that this was the reason for the unresponsiveness of the chosen RNAi pathway genes to the exogenous dsRNA exposure.

As mentioned before, RNAi in parasitic nematodes is not believed to be an accurate genetic tool for the functional analysis of their genome, due to the inconsistency of the silencing effect in those organisms (Britton and Murray, 2006, Geldhof *et al.*, 2007). Although the selected RNAi pathway genes were not appropriate to be used as internal controls that would indicate activation of the RNAi pathway, the optimisation of the soaking protocol and the finding of a successful gene silencing after 1 hour of soaking were used in the further experiments in the parasitic nematode, *T. circumcincta* (Chapter 4).

Chapter 3: An analysis of the Activation-associated Secreted Proteins in *T. circumcincta*

3.1 Introduction

Parasites have developed strategies to evade host immune responses and establish and develop in their host environment. These strategies include the excretion/secretion (ES) of various molecules (for example, proteases, acetylcholinesterases, superoxide dismutases, orthologues of host immune molecules), which help the parasites to survive and feed in their host (Knox, 2000, Schmid-Hempel, 2008). The establishment of parasites could be prevented by vaccinating animals with an essential ES component(s) and stimulating immune responses which block its function (Craig *et al.*, 2006). Activation-associated Secreted Proteins (ASPs), so called because they were initially described in the ES products of the nematode *Ancylostoma caninum* as the infective third stage larvae resumed development on entry to the definitive host (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999), and are one of the largest nematode-specific group of proteins (Parkinson *et al.*, 2004). ASPs belong to the SCP/Tpx-1/Ag5/PR-1/Sc7 protein family (SCP/TAPS; Pfam accession number: PF00188) and these family members belong to the cysteine-rich secretory protein (CRISP) superfamily (Chalmers *et al.*, 2008). Three types of ASPs have been described to date; long double-domain ASPs (DD) which are composed of two distinct, but related, domains (Hawdon *et al.*, 1996, Schallig *et al.*, 1997b, Hawdon *et al.*, 1999), short single-domain ASPs which show similarity to the C-terminal of the double-domain type (C-type SD) (Hawdon *et al.*, 1996, Schallig *et al.*, 1997b, Hawdon *et al.*, 1999) and short single-domain ASPs which show similarity to N-terminal of the double-domain type (N-type SD) (Geldhof *et al.*, 2003).

Although the precise function of the ASPs has not been revealed yet, there have been several suggestions regarding their potential function (Lu *et al.*, 1993, Hawdon *et al.*, 1996, Hawdon *et al.*, 1999, Tawe *et al.*, 2000, Geldhof *et al.*, 2003, Wang and Kim, 2003, Zhan *et al.*, 2003, Asojo *et al.*, 2005). It has been proposed that they may play a key role in the transition of nematode larvae from the free-living

state to parasitism (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999). Others suggest that they may have a role in manipulating host immune responses (Asojo *et al.*, 2005); in the parasites' reproduction (Geldhof *et al.*, 2003); in the maintenance and/or exit of the parasites from an arrested development phase (Wang and Kim, 2003); or in maintenance in their niche inside the host (Zhan *et al.*, 2003). Finally, it has been suggested that the ASPs may be key in pathogenesis, because they are homologous to venom allergens from wasps (Ag5) and ants (Ag3), they are allergenic and they induce mast cell and IgE responses, which are characteristics of anti-helminth immune responses (Lu *et al.*, 1993, Tawe *et al.*, 2000). In general, they are considered to be virulence factors that manipulate the host immune response and contribute to parasite survival in the host (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999, Asojo *et al.*, 2005).

ASPs have been isolated from various invertebrates, such as arthropods, and helminths, including *Teladorsagia circumcincta* (Cantacessi *et al.*, 2009). Nisbet *et al.* (2008) conducted a stage-specific gene expression study using cDNA from exsheathed L3 (xL3) and L4 *T. circumcincta*. In this study, 43 contigs were identified which were formed by 99 expressed sequence tags (ESTs) and encoded ASP molecules (Nisbet *et al.*, 2008). These ASP molecules included proteins of the three different types mentioned above (Nisbet *et al.*, 2008). Moreover, the majority of these ASPs were expressed in the L4 stage of the parasite, but not in the xL3 which had been exsheathed by sodium hypochlorite (NaClO) in this study (Nisbet *et al.*, 2008). Another study showed that Tci-ASP-1, an N-type SD ASP, is highly immunogenic and its recombinant form (rTci-ASP-1; expressed in *Escherichia coli*) was found to be a target of IgA responses in previously infected ewes (Nisbet *et al.*, 2010b). These results led Nisbet *et al.* (2013) to include this protein in a recombinant protein 'cocktail' vaccine against *T. circumcincta*. The vaccine contained eight proteins and was shown to induce significant protection in vaccinated compared to control animals against challenge (Nisbet *et al.*, 2013).

The ASPs have been evaluated as vaccine candidates against a number of other parasites. In the closely related parasite *O. ostertagi*, vaccination with an ASP-enriched native extract from ES of the adult stage of the parasite induced protective

immunity in cattle and reduced faecal egg counts by 60% (Geldhof *et al.*, 2002), 56% (Geldhof *et al.*, 2004) or 62% (Meyvis *et al.*, 2007). The major components of this extract were the N-type SD ASPs Oo-ASP1 and Oo-ASP2 (Geldhof *et al.*, 2002, Geldhof *et al.*, 2004, Meyvis *et al.*, 2007). Nevertheless, when a recombinant version of Oo-ASP1 (expressed in insect cells) was tested in a vaccine trial, it was not found to be protective (Geldhof *et al.*, 2008). Other species that native ASPs were evaluated in vaccine trials include: *H. contortus* (Schallig *et al.*, 1997a, Schallig and Van Leeuwen, 1997, Kooyman *et al.*, 2000) and *A. caninum* (Ghosh *et al.*, 1996, Sen *et al.*, 2000, Goud *et al.*, 2004). In *H. contortus*, the vaccinated animals showed a mean reduction of 99.9% in faecal egg counts and of 97.6% in abomasal worm burden (Schallig and Van Leeuwen, 1997); or a significant reduction (> 70%) in mean faecal egg counts and abomasal worm burden (Schallig *et al.*, 1997a); or a significant protection of 83% in 9 month-old sheep (Kooyman *et al.*, 2000) compared to the non-vaccinated controls and the adjuvant controls. In *A. caninum*, the significant protection levels that were observed in the different studies after vaccination were 79% (Ghosh *et al.*, 1996) and 58% (Sen *et al.*, 2000, Goud *et al.*, 2004).

In this chapter, the ASPs of *T. circumcincta* are characterized comprehensively because of their potential importance in inducing host protective immune responses and since there were no such studies described in the literature. A detailed bioinformatics analysis was conducted to find ASPs that are present in the *T. circumcincta* transcriptome. To further investigate the potential role of these proteins as vaccine candidates, stage-specific end-point PCR was performed to examine the transcription pattern of a selection of ASP genes. Finally, the characterization of the ASP genes was used to select the target genes for the application of RNAi in *T. circumcincta* (Chapter 4).

3.2 Materials and methods

3.2.1 Searching for SCP-like extracellular proteins in parasitic nematodes' transcriptome

The database of Nembase4 was searched for the SCP-like extracellular protein domain in all the nematode parasites by using the protein domain identifier online tool (http://www.nematodes.org/nembase4/domSearch.shtml). The database was searched against the 'IPR014044' domain ID, which represents the SCP-like extracellular protein motif.

3.2.2 SCP-like extracellular proteins in *T. circumcincta*

In order to narrow down the list of sequences with the SCP-like motif in the *T. circumcincta* database, the sequences were searched for a signal peptide by using the SignalP 4.1 online server with default settings (http://www.cbs.dtu.dk/services/SignalP/). Sequences with a signal peptide were used for a tBLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search against the nucleotide collection in order to have an indication for the type of the ASP protein. Finally, the sequences were used to build a phylogenetic tree with the online tool 'phylogeny pipeline' (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi). The default settings were chosen and the branches with values below 50% were collapsed. The yeast protein YJL079C of the allergen V5 / testis specific-1 protein family was used as an outgroup. A recent proteomics study revealed that the dominant proteins in *H. polygyrus*' ES products are the venom allergen-like (VAL) proteins (Hewitson *et al.*, 2011). As a result, *T. circumcincta*'s ASP sequences and *H. polygyrus* VAL sequences were used to build another phylogenetic tree with the same settings and outgroup as before to examine which of those proteins group together.

3.2.3 Comparison of exsheathment methods: sodium hypochlorite (NaClO) vs. CO_2

Two protocols for *in vitro* exsheathment of *T. circumcincta* were compared, namely exsheathment in NaClO or in the presence of CO_2. NaClO exsheathment of approximately 10,000 L3 was conducted as described in the Manual of Veterinary Parasitological Laboratory Techniques; Ministry of Agriculture, Fisheries and Food; Ref. Book 418; HMSO, London, UK . Specifically, 10,000 L3 were transferred to a pointed tube in 5 ml tap water. Then, NaClO was added (750 µl Milton sterilizing fluid, 2% hypochlorite, Laboratoire Rivadis) and the sample was inverted 3 times to mix. A 100 µl sample containing L3 was observed under the microscope until all the L3 were exsheathed. The L3 were then washed three times with PBS and retained for RNA extraction. After each wash, the tubes were centrifuged at 1,000 rpm for 2 min and the supernatant PBS was discarded. CO_2 exsheathment of approximately 10,000 L3 was conducted as described in previous studies (Halliday *et al.*, 2012), and the L3 were retained for RNA extraction. In short, approximately 10,000 L3 were put in a pointed tube in 2 ml of EBSS (~5,000 L3/ml) containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 1.25µg/ml amphotericin B. The L3 were incubated in a shaking water bath at 40°C and 60 strokes/min for 15 min to activate the larvae. Then, the larvae were exposed to CO_2 by vigorous bubbling for 15 min at room temperature. A seal was used (Parafilm® M, Bemis Company, Inc.) to keep the CO_2 in the tube. The tube was incubated in the shaking water bath at 40°C and 100 strokes/min for 90 min. After this, the seal was removed for 5 sec in order to release some CO_2 and then the tube was resealed.

Once the L3 were exsheathed, total RNA was extracted from the worms using TRIzol® reagent (Life Technologies™) based on the manufacturer's instructions. Larvae were homogenised in a hand-held glass Teflon® homogenizer and the concentration of the total RNA was measured using a nanodrop spectrophotometer (Nanodrop® ND-1000 UV–Vis Spectrophotometer). The total RNA was treated with RQ1 RNase-Free DNase (Promega) to degrade any genomic DNA contamination following the manufacturer's protocol. Approximately 100 ng of the DNase-treated total RNA were used as a template in a reverse-transcriptase PCR (RT-PCR)

employing primers specific for *Tci-asp-1* (Table 3. 1). Equal loading and integrity of each total RNA preparation were verified by amplifying a fragment of the *Tci-β tubulin* gene (Table 3. 1). The SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Life Technologies™) was used following the manufacturer's instructions with the following cycling conditions: 50°C for 30 min; 94°C for 2 min; 40 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min; and a 7 min extension at 72°C. Amplification products were separated on 2% (w/v) agarose gels and visualized by staining with GelRed™ (Biotium, Cambridge Bioscience, Cambridge, UK).

3.2.4 Stage-specific end-point PCR to determine the transcriptional pattern of the ASPs with a signal peptide

The primers employed for the amplification of the ASPs with a signal peptide were designed carefully to amplify unique areas of each gene since some ASP genes show high levels of conserved sequences. The sequence of each gene was searched using Blast against the Nembase4 database to find whether there were conserved areas across other family members to design gene-specific primers outside these areas. These primers targeted unique areas of each gene and were designed to have similar melting temperatures (Tm, Table 3. 1). The genes that were targeted for amplification included: *tdc00434*, *tdc00435*, *tdc00447*, *tdc00462*, *tdc00468* (*Tci-asp-1*), *tdc00533*, *tdc00571*, *tdc00582*, *tdc00627*, *tdc00691*, *tdc00879*, *tdc00997*, *tdc01250*, *tdc01309*, *tdc01347*, *tdc01479*, *tdc02274*, *tdc02547* and *tdc02673*. The genes *tdc00694* and *tdc01271* were not included in the analysis because there were no unique regions in their sequences. The following stages of *T. circumcincta* were used for the stage-specific end-point PCR: egg; L1; sheathed L3 (sL3); exsheathed L3 (xL3) by NaClO and CO_2; L4; and adult. Total RNA was extracted from each stage using TRIzol® reagent (Life Technologies™) based on the manufacturer's instructions. Larvae were homogenised using a hand-held glass Teflon® homogenizer. The concentration of the total RNA was measured using a nanodrop spectrophotometer (Nanodrop® ND-1000 UV–Vis Spectrophotometer). Total RNA was treated with RQ1 RNase-Free DNase (Promega) to degrade any genomic DNA

contamination following the manufacturer's protocol. The DNase-treated total RNA was used for the production of cDNA. SuperScript® II reverse transcriptase (Life Technologies™) was used according to the manufacturer's guidelines. Approximately 100ng of cDNA from each stage were used as a template for each gene in an end-point PCR (RT-PCR) employing primers specific for each gene (Table 3. 1). Equal loading and integrity of each total RNA preparation were verified by amplifying a fragment of the *Tci-β tubulin* (Table 3. 1). Biotaq™ DNA Polymerase (Bioline) was used following the manufacturer's instructions with the following cycling conditions: 94°C for 5 min; 40 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min; and a 7 min extension at 72°C. Amplification products were separated on 2% (w/v) agarose gels and visualized by staining with GelRed™ (Biotium, Cambridge Bioscience, Cambridge, UK). The amplification products were extracted from the agarose gel by QIAquick gel extraction kit (Qiagen), cloned into the pGEM®-T easy vector (Promega) according to the manufacturers' instructions and sent for sequencing to eurofins (http://www.eurofinsgenomics.eu/) to confirm that the correct genes were amplified.

Table 3.1. Accession numbers and primer sequences of the ASP genes and the housekeeping gene (*Tci-β tubulin*) for the stage specific end-point PCR. All the sequences are orientated form 5' to 3' end.

Gene	Accession number	Forward primer	Reverse primer
tdc00434	n/a	AACCTGCACTGGGAGAATGG	TTCCTGGCTACATCCGCATC
tdc00435	n/a	ACCGGGAATTCGGCCATTAC	AGTCGTGAAGTTCACCAAGC
tdc00447	n/a	ATTCCCTTCCGCGGCTAAGT	ACCACATGATTCCACATCCG
tdc00462	n/a	GTGCGTCGTGCTCCTTAAAT	TGTCCCCAGTTGTTCCTTTC

Gene	Accession number	Forward primer	Reverse primer
tdc00468 (*Tci-asp-1*)	FN652296.1	ATGTTCACGCCAATCGGTATT	TCAGTTTGATTTGCAAGGCTC
tdc00533	n/a	ATCGTGGCTATGGAGAGGAC	TCTCTTCGTGGTGGTAGTGG
tdc00571	n/a	ATCATCGACACATCCATGACC	AATGGACTGTTCCGAATGACG
tdc00582	n/a	TTGGTCACACTACTCCTTGG	TATCGAAGTTGCAGTGGACC
tdc00627	n/a	TACTTGACACTCTTCGTCACG	ACGTGTCTTTCTAGCAATGCC
tdc00691	n/a	AACAGTTCTGAACTCCTCAGG	TACAACACTTCGCCATCAGG
tdc00879	n/a	TGGACTGTTCTTCCATGAAGG	ACATCCTTGAGTGATTTCTGG
tdc00997	n/a	AAGTCTCTTGTGGTGGGATG	TTGATGCAGATGGAGTTTCCT
tdc01250	n/a	GGACGTAGCCATTCAAAGCC	CTCCTTTATCGAGTAATCGGC
tdc01309	n/a	GATACAGAACTGAACCTATTGC	CACACACTCGAAAGAGTGTG
tdc01347	n/a	ACGAGCATCAATGTGCTCGG	TACAGCTGGAGCTAGACATAG
tdc01479	n/a	AAGCAGGTTTCTGCTCTTCAC	CAGGCTATAAGCATTTCTTGC
tdc02274	n/a	AATCGGCTTTTCAGTGCTTCG	AGTCGGCATCTACCTTGCATG
tdc02547	n/a	AATAATGGCCAGCTTTCTCCA	TATGCCTCCATGTGCTCGAAC
tdc02673	n/a	TTCAACTGAACAGTGGATTGC	AAGTGTAGTTTCCACCATTCC

Gene	Accession number	Forward primer	Reverse primer
Tci-β tubulin	Z69258.1	TGCCACTCTTTCTGTACACC	GTTGAAGCGCGATACGCT

3.3 Results

3.3.1 Searching for SCP-like extracellular proteins in the parasitic nematodes

The results of the search for the SCP-like extracellular protein domain in all the nematode parasites by using the protein domain identifier online tool (http://www.nematodes.org/nembase4/domSearch.shtml) can be seen in Table 3. 2 and Figure 3. 1. A variety of ESTs' and clusters' numbers were identified in 40 nematode parasites.

Table 3.2. SCP-like extracellular proteins' ESTs and clusters in parasitic nematodes. Nembase4 was searched against the 'IPR014044' domain ID by using Nembase's protein domain identifier online tool. Moreover, the life cycle stages of the parasites that are indicated in the existing Nembase4 database for each parasite are mentioned in the table.

Parasite	ESTs	Clusters	Stage(s) of the parasite
Angiostrongylus cantonensis	4	2	Adult
Ancylostoma caninum	480	223	L3 and adult
Anisakis simplex	2	1	L3
Ascaris suum	4	3	L3 and adult
Ancylostoma ceylanicum	75	43	L3 and adult
Brugia malayi	11	7	L1, L3 and adult
Bursaphelenchus mucronatus	4	2	Mixed
Bursaphelenchus xylophilus	9	5	Mixed
Caenorhabditis brenneri	15	8	Mixed
Caenorhabditis briggsae	2	1	Mixed
Caenorhabditis sp. 5 AC-2008	24	11	Unknown
Ditylenchus africanus	3	2	Mixed
Dictyocaulus viviparus	56	34	Unknown

Parasite	ESTs	Clusters	Stage(s) of the parasite
Globodera pallida	4	2	Mixed
Globodera rostochiensis	8	4	L2
Heterorhabditis bacteriophora	2	1	Adult
Haemonchus contortus	17	11	Adult
Heterodera glycines	3	2	L2, L3 and L4
Heterodera schachtii	2	2	L2
Litomosoides sigmodontis	2	1	L3
Meloidogyne arenaria	14	7	Egg and L2
Meloidogyne chitwoodi	12	7	Egg and L2
Meloidogyne incognita	7	7	L2 and adult
Meloidogyne javanica	6	3	Egg
Meloidogyne paranaensis	3	3	Egg
Necator americanus	35	28	L3, L4 and adult
Nippostrongylus brasiliensis	13	10	L3 and adult
Oesophagostomum dentatum	3	3	Adult
Ostertagia ostertagi	20	13	L3, L4 and adult
Onchocerca volvulus	11	5	L3
Pristionchus pacificus	9	6	L1, L3 and mixed
Parastrongyloides trichosuri	7	6	L3 and adult
Strongyloides ratti	4	4	L3, adult and mixed
Strongyloides stercoralis	34	14	L3
Toxocara canis	12	5	Adult and unknown
Teladorsagia circumcincta	131	58	L3 and adult
Trichostrongylus vitrinus	2	2	Adult
Trichuris muris	11	7	Adult
Trichinella spiralis	1	1	L1
Wuchereria bancrofti	2	1	L3

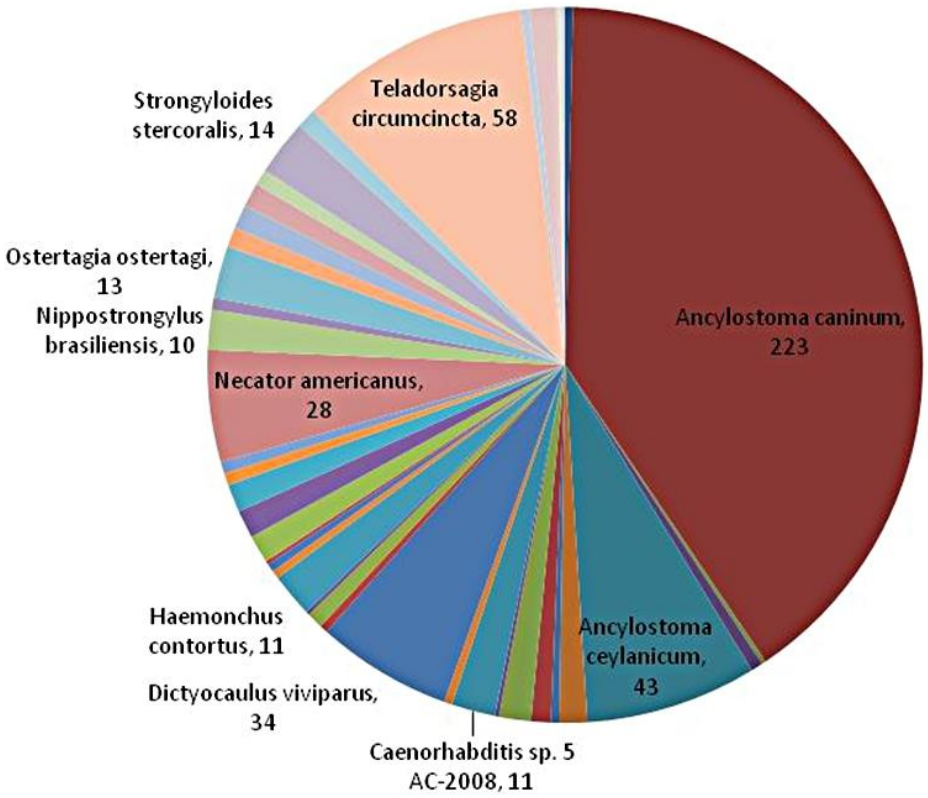


Figure 3.1. SCP-like extracellular proteins in parasitic nematodes. Species with more than 10 clusters in their transcriptome are indicated. Nembase4 was searched against the 'IPR014044' domain ID by using Nembase's protein domain identifier online tool.

3.3.2 SCP-like extracellular proteins in *T. circumcincta*

T. circumcincta has 58 clusters of genes with the SCP-like motif in the sequence. The clusters and the number of ESTs that form these clusters can be seen in Table 3. 3. Each protein encoded by the 58 genes was searched for a signal peptide. The presence (✓) or the absence (Ø) of a signal peptide can be seen again in Table 3. 3. There were 21 proteins with a signal peptide and their closest homologues and their type can be seen in Table 3. 4. Particularly, nine of these proteins were C-type SD ASPs, three N-type SD ASPs and nine double-domain ASPs. The clusters that encoded C-type SD ASPs are: *tdc00434*, *tdc00447*, *tdc00462*, *tdc00627*, *tdc00694*, *tdc01271*, *tdc01347*, *tdc02274* and *tdc02547*. The clusters that encoded N-type SD ASPs are: *tdc00435*, *tdc00468* (*Tci-asp-1*) and *tdc01479*. Finally, the

clusters that encoded DD ASPs are: *tdc00533*, *tdc00571*, *tdc00582*, *tdc00691*, *tdc00879*, *tdc00997*, *tdc01250*, *tdc01309* and *tdc02673*. The N-type SD ASPs form a distinct cluster in the phylogenetic analysis with the Oo-ASP1 and Oo-ASP2 (Figure 3. 2), which are also N-type SD ASPs. The same applies for the C-type SD ASPs, which form a cluster with the C-type SD ASPs, Hc24, Co-ASP and Oo-ASP3. The double domain ASPs formed three small clusters. Moreover, in the phylogenetic tree with the *H. polygyrus* VAL proteins, the same groupings were observed as before (Figure 3. 3). The DD proteins: Hpo-VAL-2.1 / 2.2 / 2.3, Hpo-VAL-1.2 / 1.2 / 1.4, Hpo-VAL-6, Hpo-VAL-14, Hpo-VAL-17, Hpo-VAL-12, Hpo-VAL-5 and Hpo-VAL-9 were grouped with TDC02673. Hpo-VAL-8.1 and Hpo-VAL-3.1 were grouped with the C-type SD ASPs mentioned above, despite the fact that they we described as DD in the original study (Hewitson *et al.*, 2011). There was another grouping of DD VAL proteins which included Hpo-VAL-13 with the majority of the *T. circumcincta* ASPs (Figure 3. 3). Finally, Hpo-VAL-4 was grouped with the N-type SD ASPs that was mentioned above (Figure 3. 3).

Table 3.3. ASP clusters present in *T. circumcincta*'s transcriptome. Nembase4 was searched against the 'IPR014044' domain ID. The presence or absence of a signal peptide is indicated with ✓ or Ø, respectively. Moreover, the life cycle stage(s) of the parasite that is/are indicated in the existing Nembase4 database for each cluster are mentioned in the table.

ASP cluster	ESTs	Signal peptide	Stage(s) of parasite
tdc00434	2	✓	Adult
tdc00435	8	✓	Adult
tdc00447	6	✓	Adult
tdc00454	4	Ø	Adult
tdc00460	4	Ø	Adult
tdc00462	6	✓	Adult
Tci-asp-1	7	✓	L3 and adult
tdc00487	9	Ø	Adult
tdc00533	2	✓	L3 and adult
tdc00567	1	Ø	Adult
tdc00571	2	✓	L3 and adult
tdc00582	2	✓	Adult
tdc00586	1	Ø	Adult

ASP cluster	ESTs	Signal peptide	Stage(s) of parasite
tdc00627	2	✓	Adult
tdc00656	1	Ø	Adult
tdc00691	1	✓	Adult
tdc00694	3	✓	L3 and adult
tdc00770	1	Ø	Adult
tdc00787	2	Ø	Adult
tdc00879	4	✓	Adult
tdc00942	4	Ø	Adult
tdc00969	1	Ø	Adult
tdc00997	2	✓	L3 and adult
tdc01012	1	Ø	L3 and adult
tdc01138	1	Ø	Adult
tdc01144	1	Ø	L3 and adult
tdc01200	2	Ø	Adult
tdc01250	2	✓	Adult
tdc01271	3	✓	L3 and adult
tdc01309	1	✓	Adult
tdc01315	1	Ø	Adult
tdc01347	1	✓	Adult
tdc01364	2	Ø	L3 and adult
tdc01370	1	Ø	Adult
tdc01414	1	Ø	L3 and adult
tdc01479	3	✓	Adult
tdc01588	1	Ø	L3 and adult
tdc01598	1	Ø	Adult
tdc01761	1	Ø	Adult
tdc01831	1	Ø	Adult
tdc02274	2	✓	Adult
tdc02395	1	Ø	L3
tdc02407	1	Ø	L3
tdc02486	1	Ø	L3
tdc02547	2	✓	L3
tdc02610	1	Ø	L3
tdc02632	4	Ø	L3
tdc02639	2	Ø	L3
tdc02673	2	✓	L3
tdc02686	1	Ø	L3
tdc02724	2	Ø	L3
tdc02881	2	Ø	L3
tdc02887	2	Ø	L3
tdc02933	2	Ø	L3
tdc02950	2	Ø	L3
tdc02986	2	Ø	L3

ASP cluster	ESTs	Signal peptide	Stage(s) of parasite
tdc02996	1	Ø	L3
tdc03025	2	Ø	L3

Table 3.4. *T. circumcincta* ASP clusters identified in Nembase4 that have a signal peptide, their closest homologues and their type, i.e. C-type single domain (C-type SD), N-type single domain (N-type SD) and double domain (DD).

ASP cluster	Closest homologue	E-value	ASP type
tdc00434	*O. ostertagi* ASP-3	6e-89	C-type SD
tdc00435	*O. ostertagi* ASP-2	9e-114	N-type SD
tdc00447	*O. ostertagi* ASP-3	2e-82	C-type SD
tdc00462	*O. ostertagi* ASP-3	3e-146	C-type SD
Tci-asp-1	*O. ostertagi* ASP-2	1e-105	N-type SD
tdc00533	*O. ostertagi* ASP-4	7e-163	DD
tdc00571	*O. ostertagi* ASP-4	4e-17	DD
tdc00582	*A. ceylanicum* ASP-1	3e-04	DD
tdc00627	*H. contortus* 24 kDa excretory/secretory protein	7e-91	C-type SD
tdc00691	*A. caninum* ASP-5	7e-34	DD
tdc00694	*O. ostertagi* ASP-3	5e-91	C-type SD
tdc00879	*H. polygyrus* ASP-2	4e-19	DD
tdc00997	*H. polygyrus* ASP-2	6e-18	DD
tdc01250	*O. ostertagi* ASP-4	1e-32	DD
tdc01271	*O. ostertagi* ASP-3	1e-62	C-type SD
tdc01309	*C. elegans* VAP (Venom Allergen-like Protein)-1	2e-08	DD
tdc01347	*H. contortus* 24 kDa excretory/secretory protein	3e-87	C-type SD
tdc01479	*O. ostertagi* ASP-2	1e-57	N-type SD
tdc02274	*O. ostertagi* ASP-3	7e-74	C-type SD
tdc02547	*H. contortus* 24 kDa excretory/secretory protein	6e-61	C-type SD
tdc02673	*H. polygyrus* ASP-5	5e-06	DD

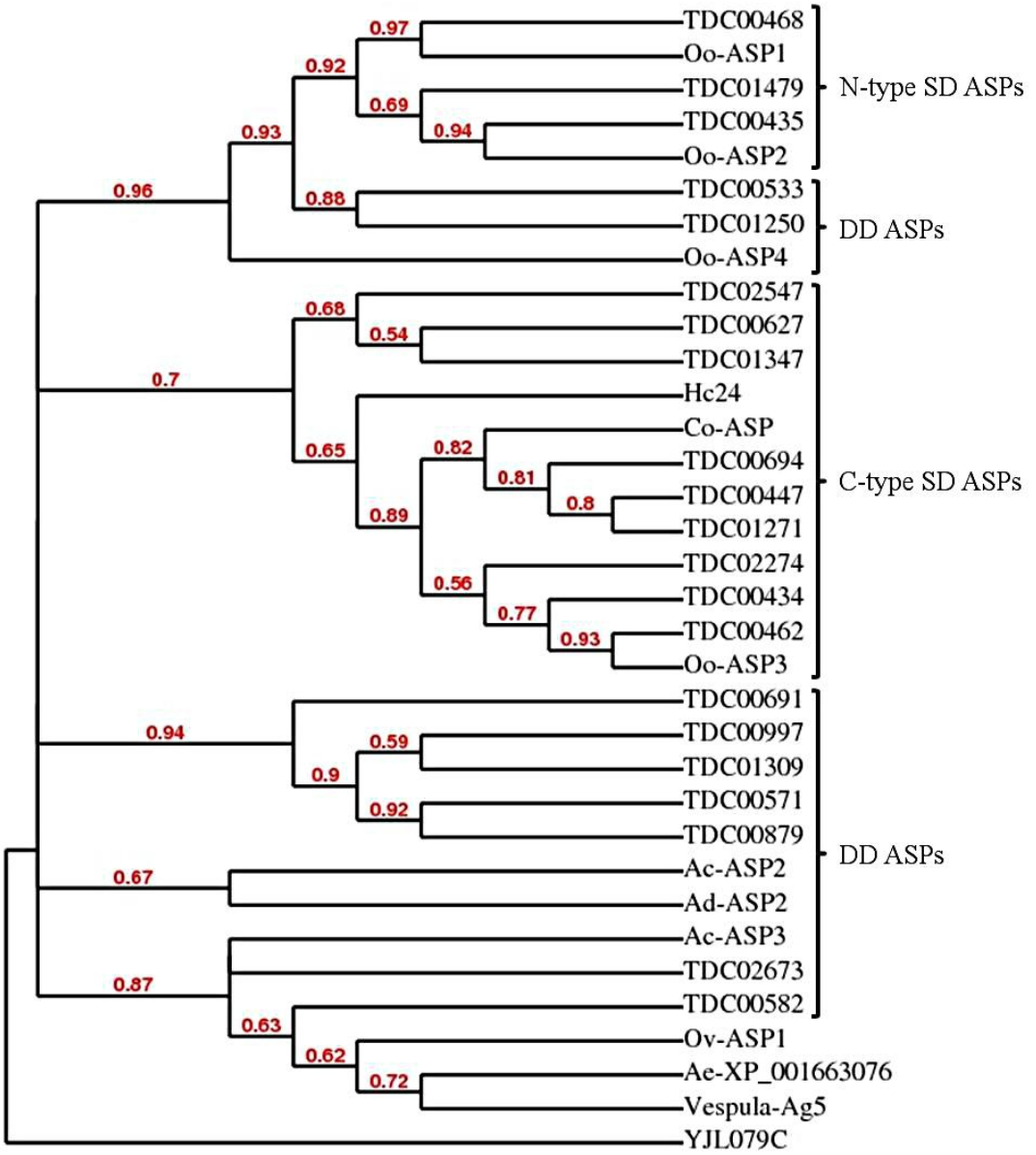
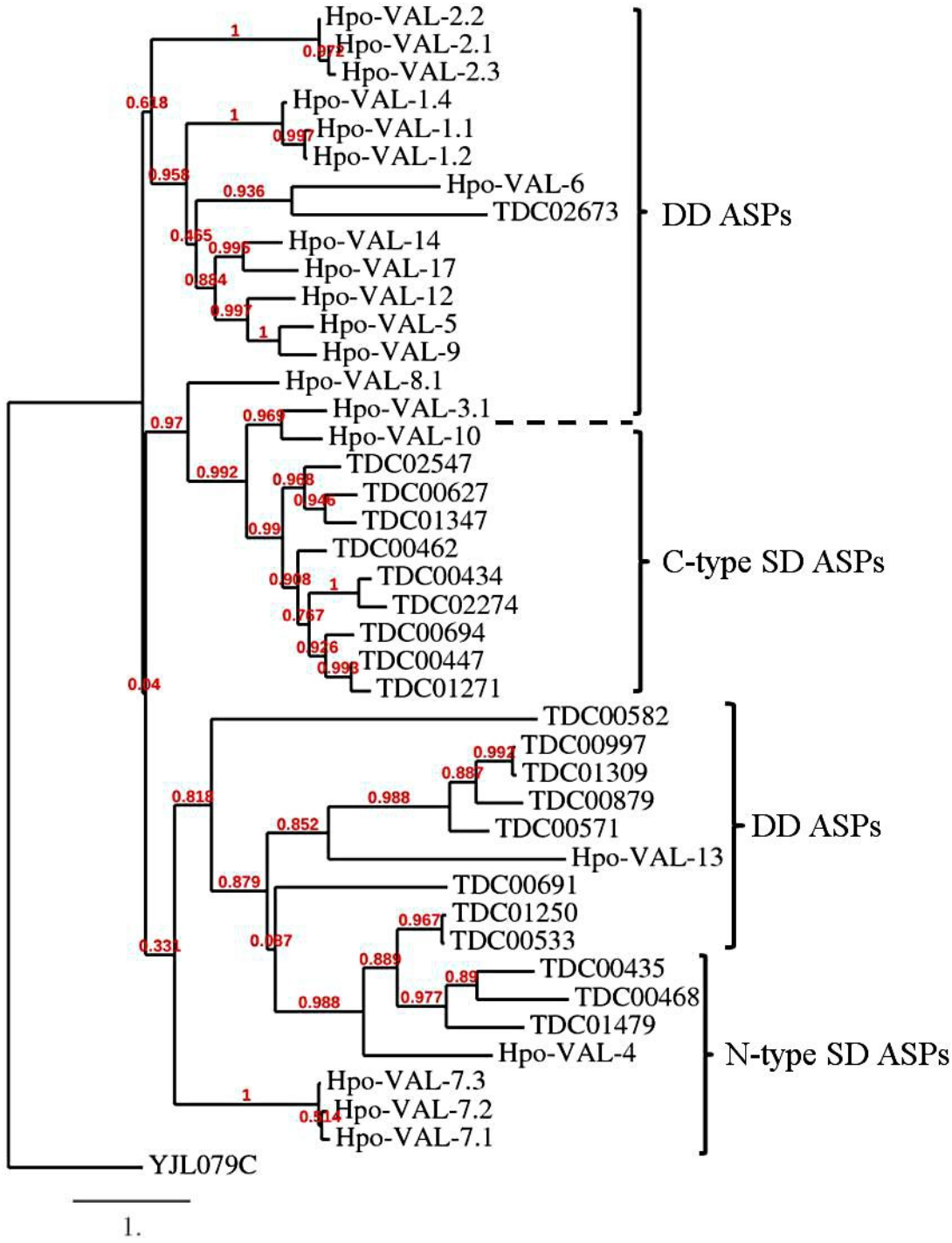


Figure 3.2. Phylogenetic tree of *T. circumcincta*'s ASP clusters that have a signal peptide in their sequence. The phylogenetic tree was constructed using the online tool 'phylogeny pipeline' (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi). The default settings were chosen and the branches with values below 50% were collapsed. The yeast protein YJL079C of the allergen V5 / testis specific-1 protein family was used as an outgroup.



3.3. Phylogenetic tree of *T. circumcincta*'s ASP clusters that have a signal peptide in their sequence and *H. polygyrus*' VAL proteins. The phylogenetic tree was constructed using the online tool 'phylogeny pipeline' (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi). The default settings were chosen and the yeast protein YJL079C of the allergen V5 / testis specific-1 protein family was used as an outgroup.

3.3.3 Comparison of exsheathment methods (NaClO and CO_2) and their effect on detection of *Tci-asp-1*transcript by RT-PCR

Comparison of the two exsheathment methods showed that the transcription of *Tci-asp-1* was activated only after exsheathing larvae using CO_2 (Figure 3. 4).

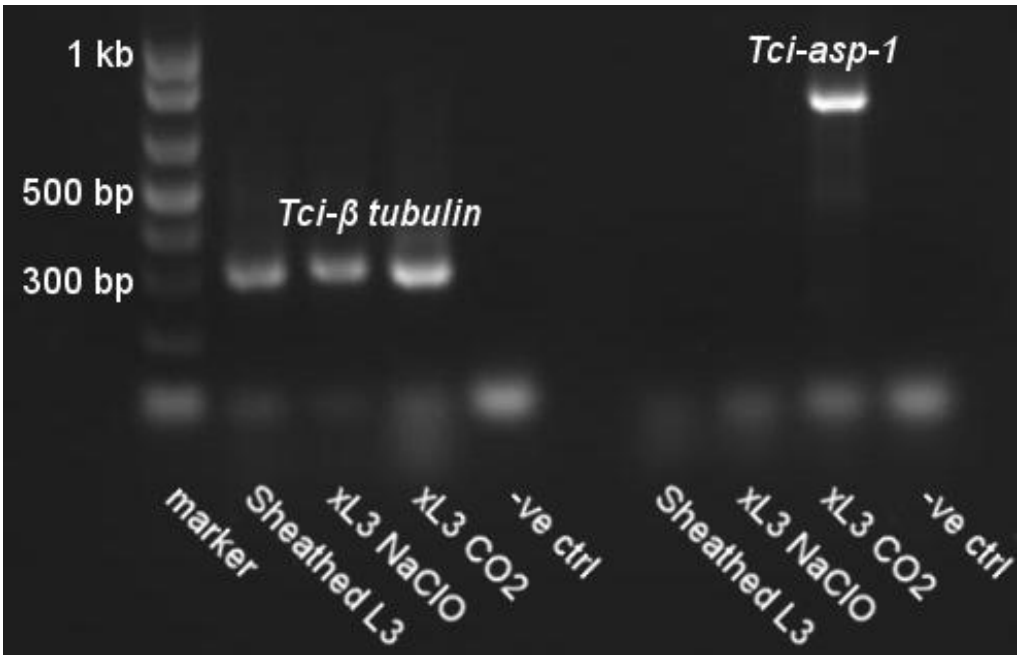


Figure 3.4. RT-PCR detection of transcript of *Tci-asp-1* and *Tci-β tubulin* of sheathed L3, L3 exsheathed using NaClO (xL3 NaClO) and L3 exsheathed using CO_2 (xL3 CO2). In each sample, 10,000 L3 *T. circumcincta* were used and *Tci-b tubulin* was used as a housekeeping gene. No template controls were included (-ve ctrl). Exsheathment by CO_2 activates the transcription of *Tci-asp-1*. The first lane shows a 1 kb DNA molecular weight marker (marker).

3.3.4 Stage specific PCR

Only 12 out of the 19 genes that were targeted here could be amplified. There were no PCR products that could be detected for the remaining 7 genes. The transcriptional patterns among the genes varied (Figure 3. 5). There were genes that were transcribed only in the parasitic stages (xL3, L4 or adults), i.e. clusters *tdc00462*, *tdc00468* (*Tci-asp-1*), *tdc00533*, *tdc00571*, *tdc00879*, *tdc01347* and

tdc02547. On the other hand, there were genes that were transcribed in both the free-living and parasitic stages of the parasite, i.e. clusters, *tdc00627*, *tdc00691*, *tdc00997*, *tdc01479* and *tdc02274*. The clusters of the genes that could not be amplified here were: *tdc00434*, *tdc00435*, *tdc00447*, *tdc00582*, *tdc01250*, *tdc01309*, and *tdc02673*. The types of the ASPs can be seen in Figure 3. 5 and Table 3. 4.

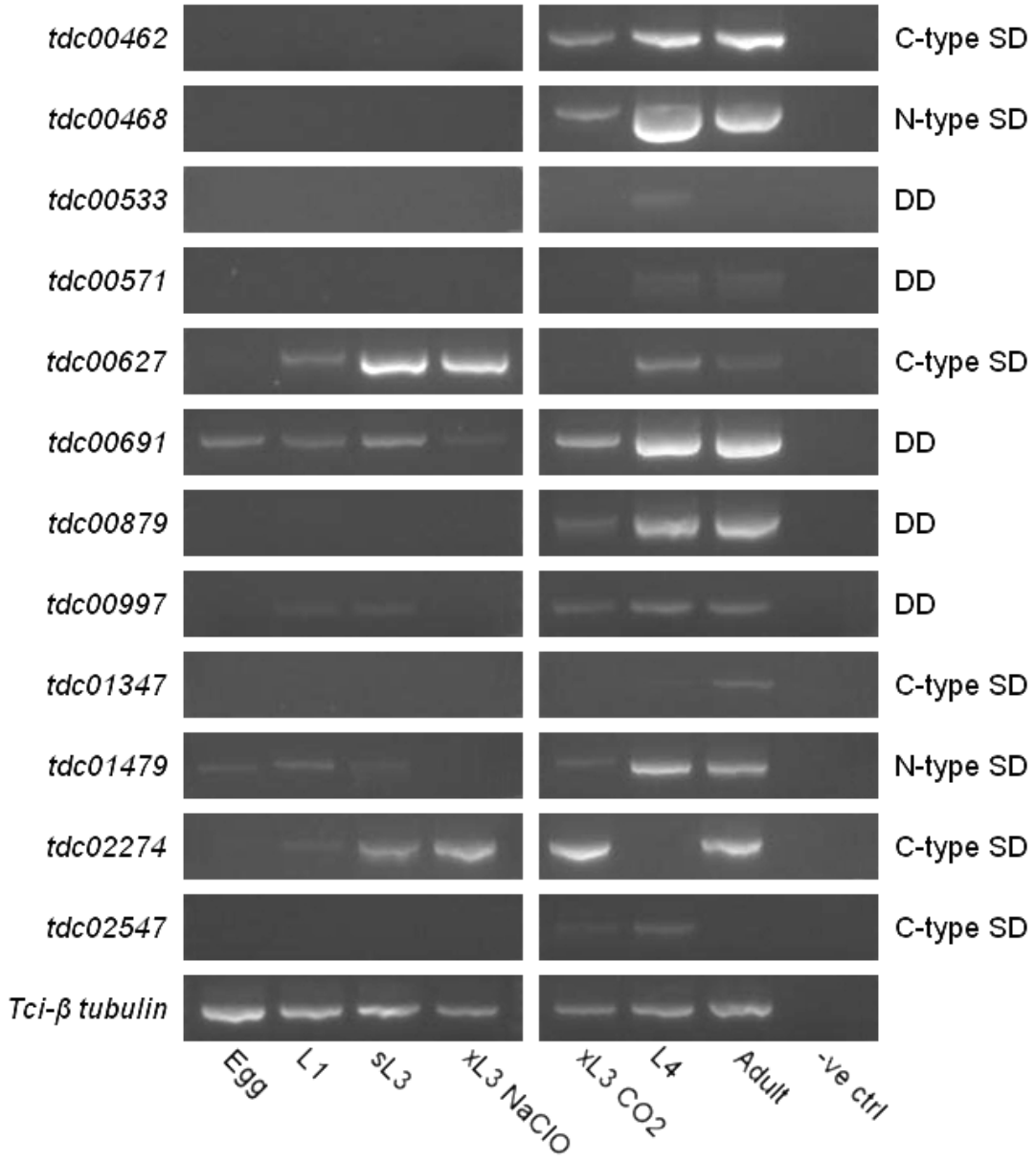


Figure 3.5. End-point PCR detection of the ASP genes with a signal peptide in the stages: egg, L1, sL3, xL3 (NaClO), xL3 (CO_2), L4 and adult. Negative (no-template) controls were included

(-ve ctrl). *Tci-β tubulin* was used as a housekeeping gene. The type of each ASP is indicated on the right-hand side of the figure: double domain ASP (DD), N-type single domain ASP (N-type SD) and C-type single domain ASP (C-type SD).

3.4 Discussion

The purpose of the experiments that were undertaken in this Chapter was to characterize the ASP molecules in *T. circumcincta*, and to investigate the relative transcription of some of these by stage-specific RT-PCR analysis. This analysis would aid the selection of target-genes for the RNAi experiments described in Chapter 4. The results have shown that at least 12 ASP molecules are expressed in the different life stages of *T. circumcincta*. Some ASPs are transcribed only in the parasitic life stages, whilst others in both the free-living and parasitic life stages. These molecules represent the three types of ASPs, i.e. double domain, C-type single domain and N-type single domain. Finally, two *in vitro* exsheathment methods for the L3 stage were compared, with CO_2 indicated as the better method of exsheathment in terms of detecting L3 transcripts that are possibly switched on at the transition to parasitism.

As mentioned before, ASPs are one of the largest nematode-specific group of proteins (Parkinson *et al.*, 2004). Although ASPs are found in great numbers in many parasitic nematodes (Table 3. 2) and have been evaluated as vaccine candidates in different species (Schallig and Van Leeuwen, 1997, Geldhof *et al.*, 2002), their precise function is yet unknown. A stage-specific gene expression analysis in *T. circumcincta* by Nisbet *et al.* (2008) revealed 6 ESTs that formed 2 contigs in the exsheathed L3 stage and 93 ESTs that formed 41 contigs in the L4 stage and encoded ASPs. These results supported earlier suggestions regarding the function of these molecules in parasitism and the number of the ASPs in the L4 stage supported the suggested role of these molecules in immune evasion and establishment (Hawdon *et al.*, 1996, Nisbet *et al.*, 2008). The search of the Nembase4 database showed that ASP genes are abundant in the *T. circumcincta* transcriptome dataset, second only to *A. caninum*. Specifically, *T. circumcincta* had 131 ESTs that formed 58 clusters. In

order to see which of these proteins were putatively excreted/secreted, the sequences were searched for a signal peptide. It was found that at least 21 out of the 58 ASP protein sequences possessed a signal peptide. However, the number of actual proteins with a signal peptide might be greater because the Nembase4 protein database is created by only sequencing parts of mRNAs. These partial sequences do not correspond to the full-length transcript and, as a result, the signal peptide might be present in regions of the genes that have not been sequenced.

Primers were designed for the sequences that encoded proteins for which a signal peptide was identified. The primers were designed so that unique regions of each transcript were amplified. Two clusters were not included in this analysis (*tdc00694* and *tdc01271*) because unique areas of sequence could not be identified in the ESTs that were available in Nembase4. Before the stage-specific PCR was performed, a comparison of exsheathment methods was done to examine whether there were any differences in transcript detection between the methods. In previous studies, transcript of *Tci-asp-1* was identified in the L4 but not in the xL3 stage of the parasite (Nisbet *et al.*, 2010b). The exsheathment in this study was stimulated by the use of NaClO (Nisbet *et al.*, 2010b). An alternative method to exsheath larvae *in vitro* is by exposure to CO_2 (Conder and Johnson, 1996). The results of the comparison here showed that the transcription of *Tci-asp-1* was activated only after exsheathing the larvae with CO_2 (Figure 3. 4). This implies that *in vitro* exsheathment with CO_2 is probably more representative of the *in vivo* situation than NaClO. If CO_2 exsheathment rather than NaClO had been used in some previous studies, more ASPs might have been identified in the xL3 stage of the parasite. In addition, this observation has implications for the outcome of transcriptional analyses of other genes in *T. circumcincta* and, quite possibly, in other parasites.

Based on the above results, cDNA was prepared from larvae exsheathed with both methods to investigate whether the CO_2 exsheathment had the same effect in genes other than *Tci-asp-1*. The results of the stage-specific end-point PCR showed that only 12 out of 19 genes that were targeted were amplified (Figure 3. 5). The transcript of several genes was identified in L3 after exsheathment with CO_2, but not with NaClO (i.e. *tdc00462*, *Tci-asp-1*, *tdc00691*, *tdc00879*, *tdc00997*, *tdc01479*,

tdc02547), which confirmed the previous observation. It is not known whether the exsheathment by CO_2 affects the transcription of other gene families except the ASPs. The sequences that were amplified represented all three types of ASPs. The C-type SD included: *tdc00462* (expressed in CO_2 xL3, L4 and adults), *tdc00627* (expressed in L1, sL3, NaClO xL3, L4 and adults), *tdc01347* (expressed in adults), *tdc02274* (expressed in L1, sL3, NaClO and CO_2 xL3 and adults) and *tdc02547* (expressed in CO_2 xL3 and L4). The N-type SD included: *Tci-asp-1* (expressed in CO_2 xL3, L4 and adults) and *tdc01479* (expressed in eggs, L1, sL3, CO_2 xL3, L4 and adults). Finally, the DD included: *tdc00533* (expressed in L4), *tdc00571* (expressed in L4 and adults), *tdc00691* (expressed in eggs, L1, sL3, NaClO and CO_2 xL3, L4 and adults), *tdc00879* (expressed in CO_2 xL3, L4 and adults) and *tdc00997* (expressed in L1, sL3, CO_2 xL3, L4 and adults). The results indicate a great variability in the transcription pattern of the genes within the three different ASP types. Thus, a potential function could not be proposed based on the type of the ASP gene. Nevertheless, the proteins encoded by the genes expressed in the parasitic stages of the parasite (xL3, L4 and adults) might play a role in the transition from the free-living to the parasitic stage and might manipulate the host's immune responses and contribute to parasite survival (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999, Asojo *et al.*, 2005). Moreover, some of these genes, such as *tdc01347*, which was found only to be transcribed in adult parasites, might play a role in reproduction (Geldhof *et al.*, 2003, Visser *et al.*, 2008). Separation of male and female parasites and investigation of transcription of this protein in the derived cDNA would inform on this possible role further (Visser *et al.*, 2008). This was not possible in this study, as the parasites used had been frozen in liquid nitrogen before and the separation of males and females was impossible. There were not any ASP genes that were found to be expressed only in the free-living stages (i.e. L1 and sL3), but there were genes that were transcribed in the free-living and parasitic stages (i.e. egg, L1, sL3, xL3, L4 or adult). It is possible that the function of these proteins be different from the other ASPs, because they are required in all the stages of the parasite.

Finally, a comparison between the transcript pattern of *T. circumcincta* ASPs and their homologues in other species has showed similarities between some of the

ASPs of the different species. In particular, *tdc00462* (expressed in CO_2 xL3, L4 and adults) had a similar though not identical expression pattern with its closest homologue *O. ostertagi-asp3* (*Oo-asp3*; expressed in L3, xL3, L4 and adults) with the former not being transcribed in the L3 stage (Visser *et al.*, 2008). Likewise, *Tci-asp-1* was transcribed in CO_2 xL3, L4 and adults, whilst its closest homologue *Oo-asp2* was found to be transcribed in L3, xL3, L4 and *adult O. ostertagi* (Geldhof *et al.*, 2003). The orthologue of *Tdc00533* (transcribed in L4) and *tdc00571* (transcribed in L4 and adults) was *Oo-asp4* (transcribed in L4) and this had the same developmental transcript pattern as *tdc00533*, but not the same as *tdc00571* (Visser *et al.*, 2008). The orthologues of *Tdc00627* and *tdc00691* cluster sequences were the *H. contortus* 24 kDa excretory/secretory protein and *A. caninum-asp5*, respectively, with both of them transcribed in the L4 and the adult stage of the parasites (Schallig *et al.*, 1997b, Zhan *et al.*, 2003). There was no information in the literature regarding the transcription pattern of *H. polygyrus*, *val-2*, the homologue of *tdc00879* and *tdc00997* (Hewitson *et al.*, 2011). The closest homologue of both *tdc01347* (adult expression pattern) and *tdc02547* (CO_2 xL3 and L4 expression) was *H. contortus* 24 kDa excretory/secretory protein (L4 and adults); and the homologues of *tdc01479* (eggs, L1, sL3, CO_2 xL3, L4 and adult expression pattern) and *tdc02274* (L1, sL3, CO_2 xL3, NaClO xL3, L4 and adult expression pattern) were *Oo-asp2* (L3, xL3L4 and adults) and *Oo-asp3* (L3, xL3, L4 and adults), respectively (Geldhof *et al.*, 2003, Visser *et al.*, 2008).

In conclusion, exsheathment of *T. circumcincta* in CO_2 is better than in NaClO for the detection of ASP genes. The bioinformatics analysis revealed 58 clusters in the *T. circumcincta* transcriptome and the stage specific PCR indicated that 12 genes that encode proteins with a signal peptide have different expression patterns. Some of the genes that were found to be expressed in the parasitic stages might be potential vaccine targets, and thus, they will be targeted with RNAi in Chapter 4. Finally, the comparison of the expression pattern of the *T. circumcincta* genes and their homologues from other species have shown variation in the transcript pattern among species, which means that the function of the proteins might be different as well.

Chapter 4: RNA interference in the parasitic nematode *T. circumcincta*

4.1 Introduction

T. circumcincta is the principal cause of ovine parasitic gastroenteritis in temperate regions and has been reported as the most predominant nematode in small ruminant flocks in the UK (Bartley *et al.*, 2003). The animals that are most affected clinically by this parasite are lambs. Teladorsagiosis causes reduced weight gain, condition loss and profuse watery diarrhoea, which leads to dehydration and emaciation of the animals and, in extreme cases, death (Scott, 2007). These symptoms not only have a major animal welfare impact, but also a substantial economic impact: it has been estimated that UK economic losses due to gastrointestinal nematode infections, of which *T. circumcincta* is the primary pathogen, exceed £84 million *per annum* (Nieuwhof and Bishop, 2005).

Administration of effective anthelmintics is the main method of control of teladorsagiosis (Scott, 2007). The major anthelmintic classes (benzimidazoles, imidazothiazoles-tetrahydropyrimidines and macrocyclic lactones) have been used frequently due to their high efficacy and their low cost (Kaplan, 2004). This approach has resulted in the development of anthelmintic resistance to one or more of these classes in several nematode species, including *T. circumcincta* (Wrigley *et al.*, 2006, Sargison, 2011). In some cases, multiple class resistance has led to some sheep farms becoming economically non-viable (Sargison *et al.*, 2007). Recently, two new drugs, monepantel (Kaminsky *et al.*, 2008b) and derquantel (Little *et al.*, 2010), were registered for use in sheep. The former belongs to the amino-acetonitrile derivative (AAD) class (Kaminsky *et al.*, 2008a) and the latter to the spiroindole (SI) class (Little *et al.*, 2010). Monepantel resistance has already been reported in *T. circumcincta* and *Trichostrongylus colubriformis* in farms in New Zealand (Scott *et al.*, 2013). In the case of derquantel, there are no reports of resistance yet, but it is likely that this will arise in future. Nowadays, anthelmintic resistance is viewed as one of the biggest problems that commercial sheep farming is facing (Besier, 2007).

As mentioned in Chapter 1, much research has focused on the development of new farm management practices, which can be used to help reduce anthelmintic use to lower selection pressure for resistance. Targeted treatments (TT) and targeted selective treatments (TST) are refugia-based strategies that are thought to decelerate the decline in efficacy of an anthelmintic and, at the same time, maintain the animal productivity (Kenyon and Jackson, 2012). With TT a group of animals within a flock is treated based on infection markers, such as faecal egg count (FEC), whilst with TST, treatment is targeted to individual animals based on parasitological (e.g. FEC), pathophysiological (e.g. diarrhoea score) and/or production (e.g. weight gain) markers (Kenyon and Jackson, 2012). Vaccination is another obvious alternative control strategy, for teladorsagiosis because protective immunity can be induced naturally or experimentally (Smith *et al.*, 1983b, Seaton *et al.*, 1989). A number of potential vaccine candidates have been identified in the Excretory/Secretory (ES) products of *T. circumcincta* (Redmond *et al.*, 2006, Nisbet *et al.*, 2009, Nisbet *et al.*, 2010a, Nisbet *et al.*, 2010b, Nisbet *et al.*, 2011). Moreover, a recent study has shown that a combination of eight recombinant antigens can stimulate significant levels of protection in vaccinated animals after experimental challenge with *T. circumcincta* L3 (Nisbet *et al.*, 2013). Other studies have tried to find novel vaccine targets against *T. circumcincta* by analyzing its transcriptome (Menon *et al.*, 2012). These bioinformatics analyses revealed more than 300 putatively secreted proteins, which included among others: ASP-like proteins, cathepsin B-like cysteine proteases and cathepsin L-like cysteine proteases (Menon *et al.*, 2012).

The results of the studies mentioned above illustrate that datasets with potential vaccine candidates are expanding and rational protocols such as *in vitro* and *in vivo* RNA interference (RNAi) might help in the further selection of appropriate vaccine candidates. It has been shown that *in vivo* RNAi, i.e. where animals are experimentally infected with parasite larvae which have been exposed to gene-specific double stranded (ds) RNA encoding the potential target gene, might be a valid method to determine the function of essential genes (Samarasinghe *et al.*, 2011). It might be possible to screen potential vaccine candidates by infecting animals with RNAi-treated larvae and observing the impact on the outcome of the

infection (Samarasinghe *et al.*, 2011). As mentioned in previous chapters, RNAi is activated by gene-specific dsRNA and results in specific gene silencing (Fire *et al.*, 1998). Variability in results been observed in the past with parasitic nematodes (Britton and Murray, 2006, Geldhof *et al.*, 2007) and for this reason attempts were made earlier in this study (Chapter 2) to define internal controls that would indicate the activation of the RNAi pathway. Here, efficacy of *in vitro* RNAi in *T. circumcincta* was evaluated by targeting genes that encode proteins found in larval ES products which are vaccine candidates (Nisbet *et al.*, 2013). The majority of the targets belong to the Activation-associated Secreted proteins (ASP) group. Other targets here include a macrophage migration inhibitory factor-like protein and a surface-associated antigen (*Tci-mif-1* and *Tci-saa-1*, respectively). These genes encode proteins that were components of the recombinant vaccine that was recently tested in sheep (Nisbet *et al.*, 2013).

4.2 Materials and methods

4.2.1 *T. circumcincta* material for RNAi experiments

Exsheathed third stage *T. circumcincta* larvae (xL3) were used to test the efficacy of RNAi. The larvae were recovered from sheep experimentally infected with MTci2 (an anthelmintic-susceptible laboratory isolate from Moredun Research Institute) using the Baermann technique (Ministry of Agriculture, Fisheries and Food; Ref. Book 418; HMSO, London, UK; 1986). The donor sheep were harnessed and a bag attached to them for 24 hours to collect faeces containing the *T. circumcincta* eggs. After this period, faeces were put in a plastic tray and left at ambient temperature for 10 days. By the 10^{th} day, the parasites developed to sheathed L3 stage (sL3). The tray with the faeces and the sL3 was filled with warm tap water (~40°C) and left at room temperature for 4 hours to allow the sL3 to migrate from the faeces into the water. After this, the water containing the sL3 was transferred to a plastic container and left overnight at room temperature for the sL3 to sediment to the bottom of the container. Finally, the majority of the supernatant was discarded and the remaining aliquot containing the sL3 transferred to a smaller container, the bottom of which was made from three layers of paper nappy liners. The container was then left to float in a funnel with a short piece of tubing to the stem containing

warm tap water (~40°C). The sL3 were left for 24 hours to migrate through the filter and sink at the bottom of the funnel. The following day the sL3 were drawn from the stem of the funnel into a cell culture flask (Corning Life Sciences) and stored at 4°C. The sL3 were exsheathed using CO_2 as described in Chapter 3. Briefly, approximately 10,000 sL3 were transferred to a pointed tube in 2 ml of EBSS (Earle's Balanced Salt Solution; Sigma®) containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 1.25µg/ml amphotericin B. The L3 were incubated for 15 min in a shaking waterbath at 40°C and 60 strokes/min to activate the larvae. Then, the larvae were exposed to CO_2 by vigorous bubbling for 15 min at room temperature. A seal was used to keep the CO_2 in the tube. The tube was then incubated for 90 min in a shaking waterbath at 40°C and 100 strokes/min. After this, the seal was removed for 5 sec in order to release some of the CO_2 and the tube was resealed. After this step, xL3 were used for the RNAi experiments.

4.2.2 Design and production of gene specific dsRNA

The majority of the RNAi target genes for *T. circumcincta* were members of the ASP family. Five ASP members were selected: *tdc00462* (dsRNA 162 bp), *Tci-asp-1* (dsRNA 218 bp), *tdc00691* (dsRNA 207 bp), *tdc00879* (dsRNA 202 bp) and *tdc01479* (dsRNA 227 bp). Additional RNAi target genes included *Tci-mif-1* (dsRNA 238 bp) and *Tci-saa-1* (dsRNA 197 bp). The selection of the ASP genes for RNAi here was based on the results of Chapter 3 (Figure 3.5) and previous studies (Nisbet *et al.*, 2010b, Nisbet *et al.*, 2013). Particularly, *Tci-asp-1*, *tdc00462* and *tdc00879* were chosen because they are transcribed in the parasitic stages (xL3, L4, adults; Chapter 3, Figure 3.5) and they represent the three different types of ASPs, i.e. N-type SD, C-type SD and DD, respectively. The remaining ASP genes (*tdc00691* and *tdc01479*) were chosen because they are transcribed in all the stages of the parasite (Chapter 3, Figure 3.5) and thus represent genes that are also transcribed in stages prior to exsheathment. Tci-mif-1 and Tci-saa-1 were chosen based on the results from previous studies (Zhan *et al.*, 2004, Nisbet *et al.*, 2009, Nisbet *et al.*, 2010a Nisbet *et al.*, 2013).First, the sequence of each gene was

searched against *T. circumcincta*'s transcriptome database (http://www.nematodes.org/nembase4/) to find unique areas for the dsRNA design. Then, it was confirmed that the unique areas did not contain the restriction sites for *Sac I* and *Sma I* and primers were designed to amplify specific gene fragments with the forward and the reverse primers having the *Sac I* (GAGCTC) and the *Sma I* (CCCGGG) restriction site at the 5' prime end, respectively (Table 4. 1). The sequence specific gene fragments were amplified from xL3 mRNA by reverse-transcriptase PCR (RT-PCR) using SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Life Technologies™) based on the manufacturer's instructions with the following cycling conditions: 50°C for 30 min; 94°C for 2 min; 40 cycles of 94°C for 30 seconds, 56°C for 1 min and 72°C for 2 min; and a 7 min extension at 72°C. Amplification products were cloned into the L4440 plasmid vector after it was digested with the restriction enzymes *Sac I* and *Sma I* and the plasmids sequenced to confirm that they had the correct insert. Finally, the plasmids were linearised using the restriction enzymes *Sac I* and *Sma I* separately and the dsRNA was produced from these templates by *in vitro* transcription using the T7 Ribomax Express RNAi System (Promega) according to the manufacturer's instructions.

Table 4.1. Accession numbers and primer sequences of the target genes for the double-stranded RNA production. All the sequences are orientated from the 5' to 3' end.

Gene	Accession number	Forward primer	Reverse primer
tdc00462	n/a	GAGCTCGGCAAATGTTCGTCGACA	CCCGGGATATGAGGAATGCTCGAA
Tci-asp-1	FN652296.1	GAGCTCAATGTTCTCGGTCCAGCT	CCCGGGAGATTACGTAGTGATGCA
tdc00691	n/a	GAGCTCTTTGTAATCGAAGCGTCG	CCCGGGAAATCTCGGAGAGGCAAC
tdc00879	n/a	GAGCTCTAATCGGTTACGGTTCAC	CCCGGGTAGATCCGTCGAGCTTCC

Gene	Accession number	Forward primer	Reverse primer
tdc01479	n/a	GAGCTCGATCCAAACTGAGAAGGC	CCCGGGTATTTTCAGTTAGATTGG
Tci-mif-1	FN599526.1	GAGCTCATCTCCTCAGTAGTCGC	CCCGGGTATCGATGAATTCAATG
Tci-saa-1	AM988835.1	GAGCTCGAGAAGAACATTCAACGA	CCCGGGTTCGCCTTGTTCTGTTCG

4.2.3 RNAi in *T. circumcincta*

Approximately 10,000 L3 *T. circumcincta* were exsheathed using CO_2 (as described before, Section 4.2.1) and 90 min after CO_2 exposure, the larvae were incubated for 1 hour in a shaking waterbath (40°C; 100 strokes/min) in 40 µl of EBSS containing 1 mg/ml dsRNA pre-mixed with 1 µl of Lipofectamine (Life Technologies™). In each experiment, eight soakings took place: one in *tdc00462*-, one in *Tci-asp-1*-, one in *tdc00691*-, one in *tdc00879*-, one in *tdc01479*-, one in *Tci-mif-1*-, one in *Tci-saa-1*-specific dsRNA and one in EBSS alone (Sigma®). The experiment was repeated on four occasions. After the end of the incubation period, total RNA was extracted from the xL3 using TRIzol® reagent (Life Technologies™) based on the instructions of the manufacturer. Larvae were homogenised in a hand-held glass Teflon® homogenizer. The concentration of the total RNA was measured using a nanodrop spectrophotometer (Nanodrop® ND-1000 UV–Vis Spectrophotometer). Finally, total RNA was treated with RQ1 RNase-Free DNase (Promega) to degrade any contaminating genomic DNA according to the manufacturer's protocol.

Approximately 100 ng of DNase treated total RNA were used as a template in RT-PCR assays employing primers specific for the RNAi targets (Table 4. 2). Equal loading and integrity of each total RNA preparation were verified by amplifying a fragment of the *T. circumcincta* β-tubulin gene (*Tci-β tubulin*; Table 4. 2). SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase

(Life Technologies™) was used based on the manufacturer's instructions with the following cycling conditions: 50°C for 30 min; 94°C for 2 min; 40 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min; and a 7 min extension at 72°C. Amplification products were separated on a 2% (w/v) agarose gel and visualized by staining with GelRed™ (Biotium, Cambridge Bioscience, Cambridge, UK). The amplification products were extracted from the agarose gel by QIAquick gel extraction kit (Qiagen), cloned in pGEM®-T easy vector (Promega) according to the manufacturers' instructions and sent for sequencing to eurofins (http://www.eurofinsgenomics.eu/) to confirm that the correct genes were amplified. Finally, a bioinformatics search was made to confirm the size of the PCR products in case samples were contaminated with genomic DNA.

Table 4.2. Accession numbers and primer sequences of the RNAi target genes and the housekeeping gene (*Tci-β tubulin*) for the end-point RT-PCR. All the sequences are orientated from the 5' to 3' end.

Gene	Accession number	Forward primer	Reverse primer
tdc00462	n/a	GTGCGTCGTGCTCCTTAAAT	TGTCCCCAGTTGTTCCTTTC
Tci-asp-1	FN652296.1	ATGTTCACGCCAATCGGTATT	TCAGTTTGATTTGCAAGGCTC
tdc00691	n/a	AACAGTTCTGAACTCCTCAGG	TACAACACTTCGCCATCAGG
tdc00879	n/a	TGGACTGTTCTTCCATGAAGG	ACATCCTTGAGTGATTTCTGG
tdc01479	n/a	AAGCAGGTTTCTGCTCTTCAC	CAGGCTATAAGCATTTCTTGC
Tci-mif-1	FN599526.1	CGGTTTTCTCATTCCACACC	TTCTGCTGTTGAAGGCAATG
Tci-saa-1	AM988835.1	GGATTTTTCGATGAC	GAGTGAACGCGAGAA

Gene	Accession number	Forward primer	Reverse primer
		GTTAGCG	TCACAG
Tci-β tubulin	Z69258.1	TGCCACTCTTTCTGTACACC	GTTGAAGCGCGATACGCT

4.2.4 Transcriptional pattern of *Tci-asp-1* and immunoblotting of somatic extracts using anti-rTci-ASP-1 sera after exsheathment by CO_2

Approximately 80,000 xL3 *T. circumcincta* were used for this experiment. Once the L3 were exsheathed by CO_2 exposure (see Section 4.2.1); 90 min after the CO_2 exposure they were divided into four batches of ~20,000 L3. The larvae were incubated in 4 ml EBSS (Sigma®) and kept *in vitro* in a shaking waterbath (at 40°C and 100 strokes/min) for 24, 48, 72 and 96 hours. At each time point, the larvae were divided again and ~10,000 xL3 were used for total RNA extraction (TRIzol® reagent; Life Technologies™) to examine the transcript levels of *Tci-asp-1* by RT-PCR at these time-points as described in section 4.2.3. The remaining ~10,000 xL3 were used in immunoblotting experiments to examine the protein levels at the same time-points. The latter was conducted as described by Nisbet *et al.* (2010b). In short, the xL3 were washed three times with phosphate-buffered saline (PBS). After each wash, larvae were pellet by centrifugation at 1,000 rpm for 2 min and the supernatant aspirated leaving a final volume of 50µl. The samples were denatured at 95°C for 10 min after adding 19.25µl NuPAGE® LDS sample buffer (Life Technologies™) and 7.7µl NuPAGE® sample reducing agent (Life Technologies™). A total of 10µl of each somatic protein preparation were subjected to electrophoresis (200V constant voltage for 35 min) on NuPAGE® Bis-Tris 4–12% gels under reducing conditions employing NuPAGE® MES SDS running buffer (Life Technologies™) followed by staining the proteins with SimplyBlue™ SafeStain (Life Technologies™) according to the manufacturer's instructions. Subsequently, the somatic extracts along with recombinant Tci-ASP-1 (rTci-ASP-1), were subjected to immunoblotting using rabbit antibodies to rTci-ASP-1 [kindly donated by Dr Nisbet, Nisbet et al. (2010b)].

Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Life Technologies™); the membrane was washed briefly in TNTT (10 mM Tris, 0.5M NaCl, 0·05% Tween 20, 0.01% thiomersal pH 7.4) and incubated in TNTT overnight at 4°C to block any non-specific protein binding. The blots were incubated with anti-rTci-ASP-1 rabbit sera diluted (1:100) in TNTT for 1 h at room temperature with constant rocking. Negative controls were included by incubating the blots in pre-immunisation sera diluted (1:100) in TNTT for the same period. Then, the membranes were washed three times in TNTT (10 min/wash) followed by 1 hour in swine anti-rabbit IgG horseradish peroxidase (HRP) conjugate (DakoCytomation) diluted 1:1000 in TNTT; and washed again three times in TNTT (10min/wash). Peroxidase activity was revealed using 3,3′-Diaminobenzidine (DAB) as substrate.

4.2.5 *Tci-asp-1* transcript recovery after successful RNAi

An experiment was conducted to examine the duration of the silencing effect. Approximately 60,000 xL3 (see Section 4.2.1) were used. After 90 min of CO_2 exposure, L3 were divided into six separate tubes, each one containing ~10,000 L3 in 2 ml EBSS (Sigma®). Three of these were used as controls (untreated) and the remaining used for soaking the xL3 in *Tci-asp-1* specific dsRNA (treated) for 1 hour as described in Section 4.2.3. After the soaking period, the xL3 were washed three times with 1xPBS to remove the gene-specific dsRNA. The tubes were centrifuged after each wash at 1,000 rpm for 2 min and the supernatant discarded leaving a final volume of ~50µl. Finally, 2 ml EBSS (Sigma®) were added to the tubes and the larvae (treated and untreated) were cultured *in vitro* in a shaking waterbath (at 40°C and 100 strokes/min) for 24, 48 and 72 hours. At each time point, ~10,000 treated and ~10,000 untreated xL3 were used for RNA extraction using TRIzol® reagent (Life Technologies™). The concentration of the total RNA was measured using a nanodrop spectrophotometer (Nanodrop® ND-1000 UV–Vis Spectrophotometer). The total RNA was DNase treated (RQ1 RNase-Free DNase; Promega) and used as a

template in an RT-PCR as described in Section 4.2.3. This experiment was conducted in duplicate.

4.2.6 Searching for the essential RNAi pathway genes in *T. circumcincta*'s genome

A bioinformatics search was conducted to search for the RNAi pathway genes in *T. circumcincta*. This was based on the study conducted by Dalzell *et al.*, 2011. The protein sequences of 74 *C. elegans* proteins that were used by Dalzell *et al.*, 2011 and considered to be 'essential' for a functional RNAi pathway were retrieved from WormBase (www.wormbase.org; release WS243). Those sequences were used to search the *T. circumcincta* genome; tBLASTn was used against the "*T. circumcincta* phusion assembled supercontigs" and the "*T. circumcincta* unassembled sequence reads" (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/t_circumcincta). The primary BLAST hit from the *T. circumcincta* genome were subjected to reciprocal tBLASTx against the *C. elegans* nucleotide collection on the NCBI BLAST server, using default settings (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The homology of the genes was confirmed by taking under consideration both BLAST searches.

At the time of the study, the *T. circumcincta* genome was not fully annotated and, as a result, some of the RNAi pathway genes were sought in the genome of *Haemonchus contortus*, this being a closely related trichostrongylid nematode which infects sheep and goats. The protein sequences were retrieved from WormBase (www.wormbase.org; release WS243) and were used to search the *H. contortus* genome. BLASTp and tBLASTn were used against the *H. contortus* version 1 protein sequences and *H. contortus* version 1 gene sequences databases, respectively (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus).

4.2.7 Testing dsRNA delivery by soaking larvae in fluorescently labeled RNA (siRNA) and dsRNA

Approximately 1,000 xL3 were used for this experiment (see Section 4.2.1). The parasites were split into 5 aliquots (~200 larvae each), and pelleted by centrifugation at 1000 rpm for 2 min. The supernatant was removed and FITC (Fluorescein isothiocyanate) – labelled siRNA (AllStars Neg. siRNA AF 488, Qiagen) pre-mixed with 1 µl of Lipofectamine (Life Technologies™) were added to each tube at a final concentration of 1 mg/ml in a final volume of 40 µl. The tubes were covered with tin foil to protect the FITC-labelled siRNA from daylight and they were kept in a shaking waterbath at 40°C and 100 strokes per min for 1, 24, 48, 72 and 96 hours. An inverted microscope (Zeiss Axiovert 25) fitted with a UV blue-range filter (495 nm) was used to photograph the larvae at each time-point to monitor uptake of the FITC-labelled siRNA. Control, untreated, xL3 were photographed at the same time points to estimate autofluorescence. At these time points, a tube was removed from the waterbath; the worms were washed three times with 1xPBS by centrifuging (1,000 rpm for 2 min) and removing the supernatant each time. Finally, the larvae were transferred onto a glass slide (Thermo Scientific) and examined at a magnification of 20x. Pictures of the larvae were taken using a digital SLR camera (Nikon D90). Larvae with FITC-labelled siRNA visible in their bodies were considered to uptake siRNA. If the worms were moving fast and the pictures were out of focus, the worms were incubated for a short period at 4°C to slow them down. The same experiment was repeated with fluorescently labelled dsRNA. The dsRNA was sequence specific for *Tci-asp-1* (see Section 4.2.2) and was fluorescently labelled by using Silencer® siRNA labeling kit (Life Technologies™) following the manufacturer's protocol.

4.2.8 Testing the efficacy of RNAi after soaking xL3 to heterogeneous small interfering RNA (siRNA)

Heterogeneous siRNA (hsiRNA), specific for *Tci-asp-1*, was produced as described previously (Landmann *et al.*, 2012). Briefly, *Tci-asp-1* gene-specific dsRNA was produced by *in vitro* transcription using the T7 Ribomax Express RNAi

System (Promega, see Section 4.2.4). Subsequently, the dsRNA was processed to hsiRNA by using the Shortcut RNase III enzyme (New England Biolabs) according to the manufacturer's instructions. The protocol used in Section 4.2.3 was followed for the soaking. Briefly, ~10,000 L3 *T. circumcincta* were exsheathed by CO_2 (Section 4.2.1) and 90 min after the CO_2 exposure, the larvae were incubated for 1 hour in a shaking waterbath (40°C; 100 strokes/min) in 40µl of EBSS (Sigma®) containing 1 mg/ml hsiRNA pre-mixed with 1 µl of Lipofectamine (Life Technologies™). In each experiment, two soakings took place, one in *Tci-asp-1* specific hsiRNA, and one in EBSS (Sigma®). The experiment was conducted in duplicate. After the end of the incubation period, total RNA was extracted from the worms and the transcript levels of the gene examined by RT-PCR (SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase; Life Technologies™) as described in Section 4.2.3.

4.2.9 Testing the effect of storage period of the sL3 on the RNAi efficacy

Two different batches of larvae were used in this experiment. Here, sL3 were stored for a year ('old') and sL3 stored for a week ('fresh') in the lab at 4°C. The vitality and the motility of the sL3 of the two batches was the same as judged by microscopic examination. As before, the L3 were exsheathed in CO_2 and subsequently soaked for 1 hour in *Tci-asp-1* specific dsRNA (Section 4.2.3.). Controls of xL3 soaked in EBSS (Sigma®) alone were included. The experiment was conducted in quadruplicate. After the end of the incubation period, RNA was extracted from the worms and transcript levels of the genes examined by RT-PCR as before. A time course experiment took place using larvae which were stored for less than a week in the lab at 4°C. In this experiment, L3 from the same batch were soaked *Tci-asp-1* specific dsRNA on a weekly basis to examine whether and when the L3 would be refractory to RNAi.

4.2.10 Effect of RNAi with hsiRNA on 'fresh' larvae

The genes that were targeted were *Tci-asp-1*, *Tci-mif-1* and *Tci-saa-1*. The same principles were followed for the production of the gene-specific hsiRNA as detailed in Section 4.2.8. Once the hsiRNAs were produced, ~10,000 sL3 (stored for less than a week at 4°C) were exsheathed with CO_2 and the RNAi protocol was followed as in Section 4.2.8. In each experiment, seven soakings took place, one in dsRNA and one in hsiRNA specific for *Tci-asp-1*; one in dsRNA and one in hsiRNA specific for *Tci-mif-1*; one in dsRNA and one in hsiRNA specific for *Tci-saa-1*; and one in EBSS. After soaking, total RNA was extracted from the worms and transcript levels were examined by RT-PCR as described in Section 4.2.3. Equal loading and integrity of each RNA preparation were verified by amplifying a fragment of the *Tci β-tubulin* gene.

4.3 Results

4.3.1 RNAi in *T. circumcincta*

The sequencing results showed that the inserts encoded the correct sequence for each of the target genes in the plasmids used for generating the gene specific dsRNA (*tdc00462, Tci-asp-1, tdc00691, tdc00879, tdc01479, Tci-mif-1, Tci-saa-1*). After successful production of dsRNA, the RNAi experiments showed successful silencing of *tdc00462, Tci-asp-1, tdc00691* and *tdc01479* after 1 hour of soaking in gene specific dsRNA, but not for the other target genes (Figure 4. 1). Sequencing results for the RT-PCR products confirmed that the correct genes had been amplified. The bioinformatics search indicated that two of the amplification products (*Tci-asp-1, tdc01479*) would be larger if genomic DNA had been amplified at the RT-PCR step, as an intron was present between the primer binding sites. Particularly, there was a 499 bp intron in the genomic sequence of *Tci-asp-1* and a 499 bp intron in the genomic sequence of *tdc01479*. Intron information could not be found for the rest of the targets (*tdc00462, tdc00691, tdc00879, Tci-mif-1* and *Tci-saa-1*) potentially because the *T. circumcincta* genome was not fully annotated at the time of the study.

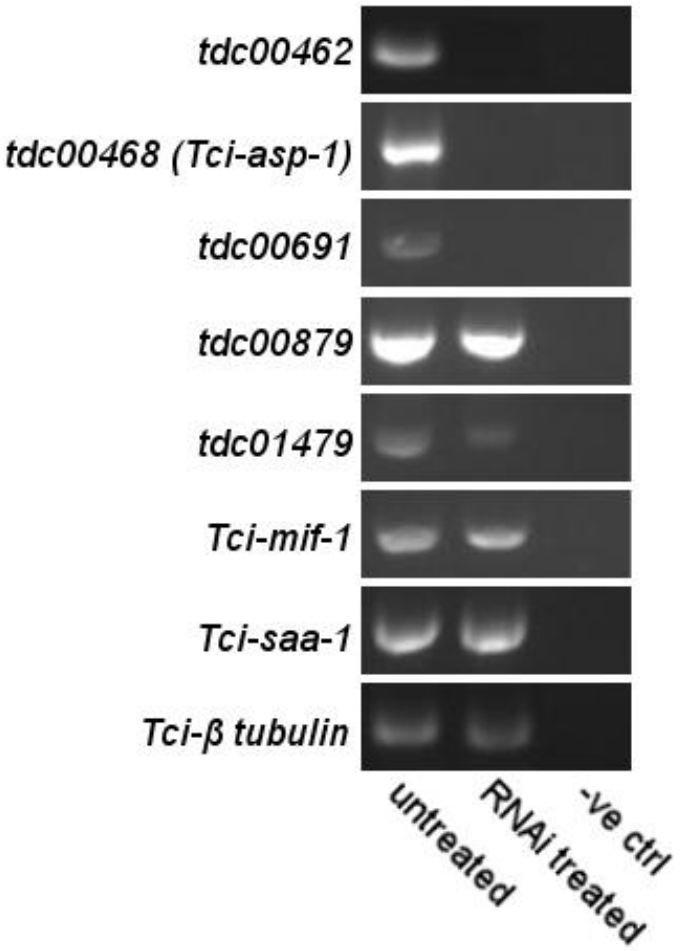


Figure 4.1. RT-PCR detection of the RNAi target genes (*tdc00462*, *Tci-asp-1*, *tdc00691*, *tdc00879*, *tdc01479*, *Tci-mif-1*, *Tci-saa-1*) transcripts in CO_2-exsheathed L3 *T. circumcincta* after soaking in gene-specific dsRNA for 1 hour (RNAi treated) compared with untreated worms (untreated). Negative no-template controls were included (-ve ctrl). *Tci-β tubulin* was used as a housekeeping gene. The transcript levels of *tdc00462*, *Tci-asp-1* and *tdc00691* were all reduced following RNAi treatment while the other genes, including the control, were unaffected.

4.3.2 Transcriptional pattern of *Tci-asp-1* and immunoblotting of somatic extracts using anti-rTci-ASP-1 sera after exsheathment by CO_2

Since RNAi was successful for only four of the ASP genes, I focused on *Tci-asp-1* for the subsequent experiments. The transcript levels of *Tci-asp-1* increased proportionally with time (Figure 4. 2) after *in vitro* maintenance of CO_2-xL3 for up to 96 h. Although the somatic extract preparations of the *in vitro* maintained xL3

were successful (Figure 4. 3), detection of the Tci-ASP-1 protein by probing with rTci-ASP-1-specific antisera indicated that the protein was not detectable by immunoblotting at these time points (Figure 4. 4).

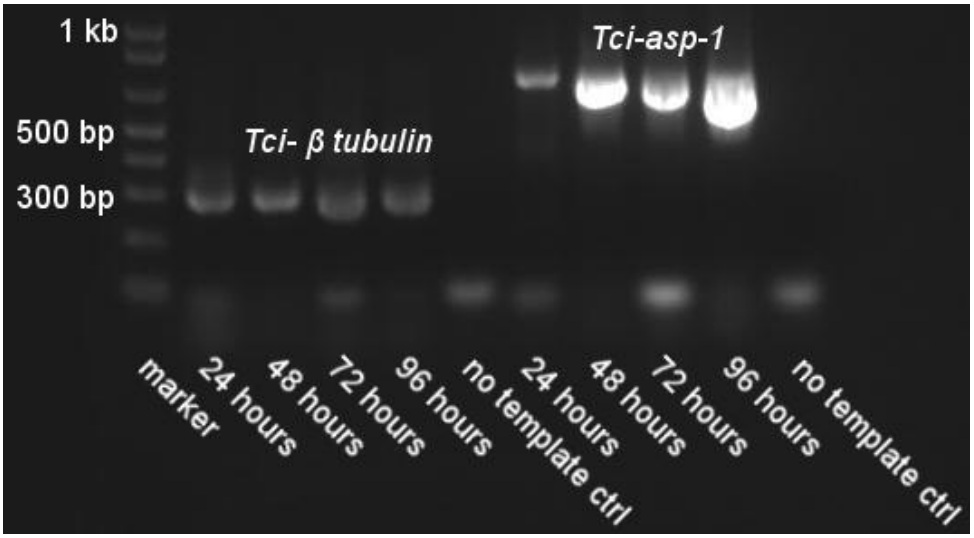


Figure 4.2. The effect of CO_2 exsheathment of L3 *T. circumcincta* on *Tci-asp-1* transcript over time. Transcript abundance increased markedly with time after *in vitro* maintenance of the exsheathed L3, for up to 96 h (lanes 7-11). *Tci- β tubulin* was used as a housekeeping gene (lanes 2-6) and 'no template' controls (lanes 6, 11) were included.

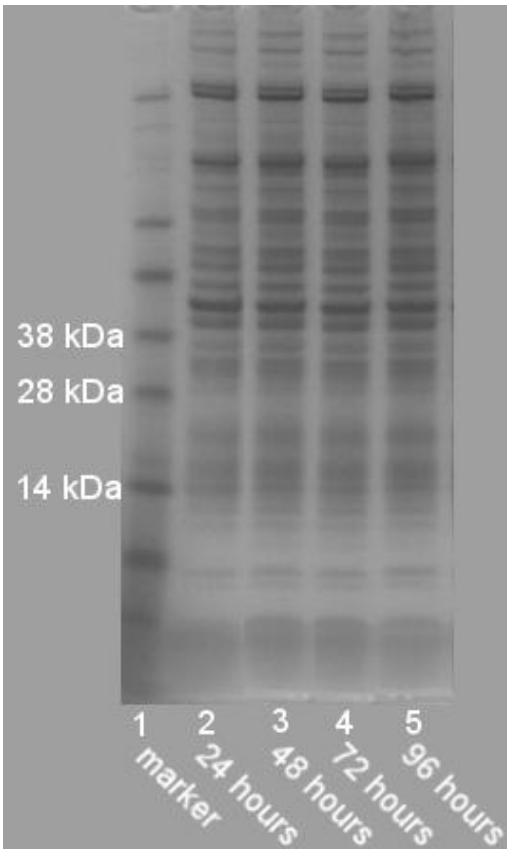


Figure 4.3. Protein profiles of the somatic extracts of L3 *T. circumcincta*. The L3 were *in vitro* exsheathed using CO_2 and maintained *in vitro* for 24, 48, 72 and 96 hours. 10,000 xL3 from each time-point were denatured at 95°C for 10 min and 10µl of each somatic protein preparation were subjected to electrophoresis under reducing conditions followed by staining the proteins with SimplyBlue™ SafeStain (Life Technologies™). The results suggested a successful extraction of the proteins from the xL3.

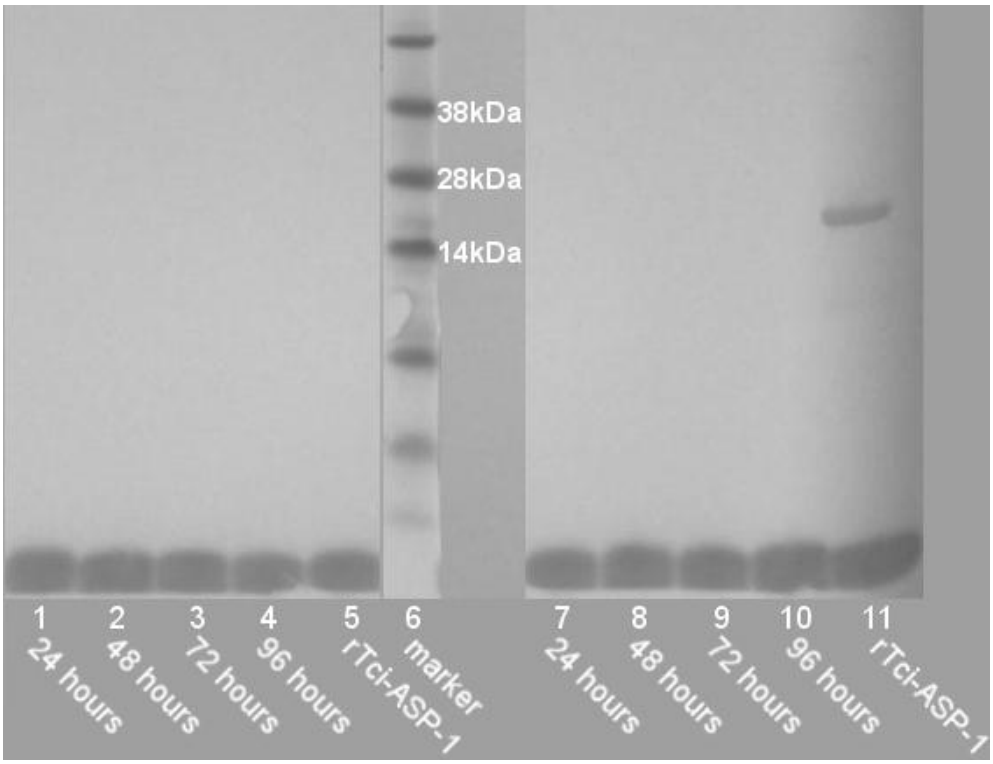


Figure 4.4. Immunoblot of the somatic extracts of xL3 *T. circumcincta* using rabbit antibodies to rTci-ASP-1. The L3 were *in vitro* exsheathed using CO_2 and maintained *in vitro* for 24, 48, 72 and 96 hours. 10,000 xL3 from each time-point were denatured at 95°C for 10 min and 10µl of each somatic protein preparation were subjected to electrophoresis under reducing conditions followed by Western blot. Tci-ASP-1 was not detected in neither of the time points (24, 48, 72 and 96 hours). Pure recombinant Tci-ASP-1 (rTci-ASP-1) was used as a positive control (lane 11). Lanes 1-5 represent the samples incubated in pre-immune serum (negative control) and lanes 7-11 the samples incubated in anti-rTci-ASP-1 serum.

4.3.3 *Tci-asp-1* transcript recovery after successful RNAi

The transcript recovery experiments indicated that *Tci-asp-1* mRNA had not recovered to the levels observed in controls, even at 24 hours after the end of soaking and the removal of the dsRNA. However, after 48 hours PCR products amplified were similar to those observed in the control samples (Figure 4. 5). After the consistently successful RNAi experiments described here and in Section 4.3.1, the silencing effect was found to be non-reproducible for subsequent experiments. An example of the unsuccessful results can be seen in Figure 4. 6.

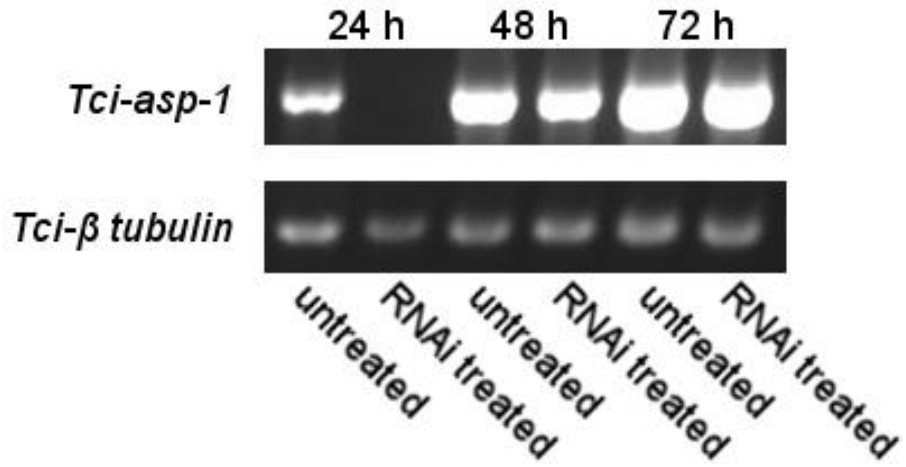


Figure 4.5. RT-PCR detection of *Tci-asp-1* transcript in CO_2-exsheathed L3 *T. circumcincta* 24, 48 and 72 hours after the end of the 1 hour soaking period and the removal of the dsRNA from the culture medium. 24 hours after RNAi in the absence of dsRNA the transcript has not recovered yet. However, after 48 hours the transcript has recovered to normal levels compared with the untreated xL3. *Tci-β tubulin* was used as a housekeeping gene.

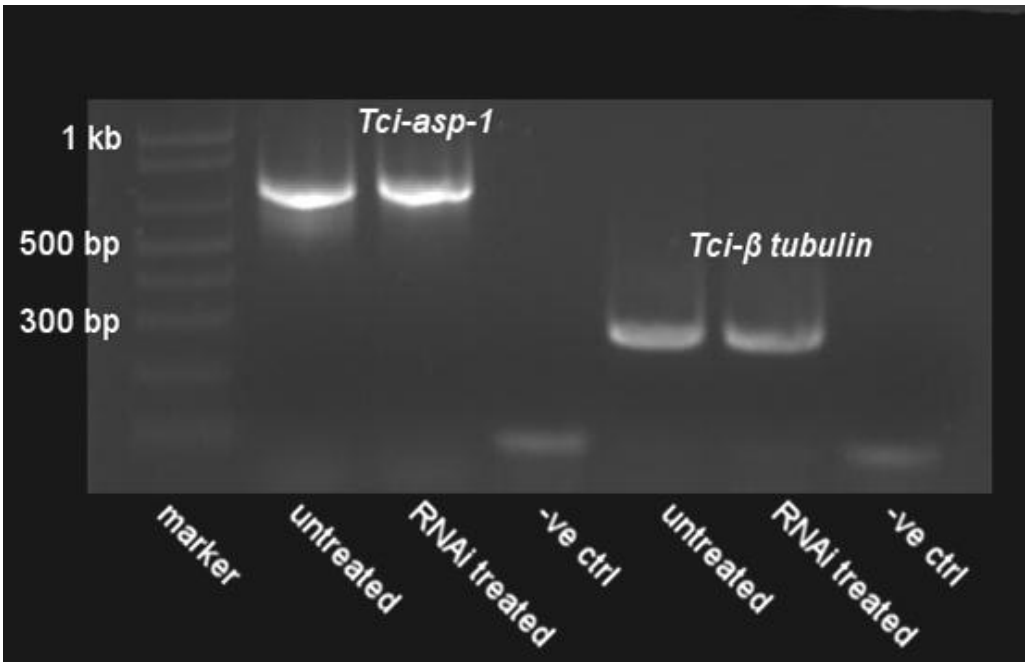


Figure 4.6. RT-PCR detection of the *Tci-asp-1* transcript in CO_2-exsheathed L3 *T. circumcincta* after soaking in *Tci-asp-1*-specific dsRNA for 1 hour (RNAi treated) compared with untreated worms. RNAi was unsuccessful as the transcript levels were the same in the treated and

untreated xL3. Negative no-template controls were included (-ve ctrl). *Tci-β tubulin* was used as a housekeeping gene.

4.3.4 Searching for potential reasons for the RNAi inconsistencies

It had been suggested that a potential reason for the RNAi inconsistencies in parasitic nematodes might be the absence of some of the RNAi pathway genes (Viney and Thompson, 2008). As a result, a bioinformatics search was conducted to search for the RNAi pathway genes in the *T. circumcincta* genome. The bioinformatics search resulted in the identification of 37 out 74 RNAi pathway genes which are thought to be 'essential' for RNAi in *C. elegans* (Table 4. 3; Dalzell *et al.*, 2011). The genes that were not identified in the *T. circumcincta* genome were sought in the *H. contortus* genome. Moreover, the RNAi pathway genes that were not identified in *H. contortus* in previous studies (Geldhof *et al.*, 2006b, Dalzell *et al.*, 2011), were also sought in the *H. contortus* genome given that this had been updated since these previous searches. In *H. contortus*, 66 of the 74 'essential' RNAi pathway genes were identified including *rde-4*. The accession number of the primary hit, the E-value and the percentage of coverage and the identities of the reciprocal tBLASTx search against the nucleotide selection of *C. elegans* in NCBI can be seen in Table 4. 4.

In order to investigate whether the delivery of dsRNA to the parasites was the factor that affected RNAi consistency, as it had been suggested previously (Viney and Thompson, 2008), I soaked xL3 in FITC-labeled siRNAs and dsRNA. The results have shown that after 1 and 24 hours of soaking, both the soaked and the control xL3 showed the same level of fluorescence. However, after 48, 72 and 96 hours of soaking, the intensity of the fluorescence in worms soaked in labelled siRNAs was higher than the level of auto-fluorescence observed in the control worms (Figure 4. 7). Likewise, soaking xL3 in FITC-labeled dsRNA revealed higher intensity of fluorescence after 48, 72 and 96 hours of soaking compared to non-

dsRNA soaked worms. The intensity of fluorescence in this case was not as strong as that of the samples soaked in siRNAs (Figure4. 7).

Finally, xL3 were soaked in hsiRNAs because it was found to be successful in *Brugia malayi* (Landmann *et al.*, 2012). Soaking xL3 for 1 hour in hsiRNA, specific for *Tci-asp-1*, did not result in knock down in these specific experiments (data not shown; image similar to Figure 4. 6). On the other hand, the comparison between 'fresh' L3 and 'old' L3 showed a successful silencing only in 'fresh' L3 (stored for a week) but not in 'old' L3 (stored for a year). This was a consistent finding in all four experiments (Figure 4. 8). The time course experiment showed that the 'fresh' L3 were susceptible to RNAi for 3 weeks after the copro-culture (Figure 4. 9).

Table 4.3. RNAi pathway genes present (✓) and absent (Ø) in the genomes of *T. circumcincta* and *H. contortus*. Marked with bold are the genes that have not been found in *H. contortus*' genome in previous studies.

	Gene	*T. circumcincta*	*H. contortus*
siRNA synthesis proteins	*drh-3*	✓	✓
	drsh-1	✓	✓
	xpo-1	✓	✓
	xpo-2	✓	✓
	dcr-1	✓	✓
	pash-1	Ø	✓
	rde-4	Ø	✓
	xpo-3	✓	✓
	drh-1	✓	✓
siRNA amplification proteins	*smg-2*	✓	✓
	smg-6	Ø	✓
	ego-1	✓	✓
	rrf-3	✓	✓
	rrf-1	✓	✓
	smg-5	✓	✓
	rsd-2	Ø	**Ø**
RNAi signal	*rsd-3*	Ø	✓

	Gene	*T. circumcincta*	*H. contortus*
spreading proteins	*sid-1*	✓	✓
	rsd-6	Ø	**Ø**
	sid-2	Ø	**Ø**
RISC proteins	***tsn-1***	✓	✓
	vig-1	Ø	✓
	ain-1[a]	✓	✓
	ain-2[a]	Ø	✓
RNAi inhibitor proteins	*eri-1*	✓	✓
	xrn-2	✓	✓
	adr-2	✓	✓
	xrn-1	✓	✓
	adr-1	✓	✓
	lin-15b	Ø	✓
	eri-5	Ø	✓
	eri-6/7	Ø	✓
	eri-3	Ø	✓
Nuclear RNAi effectors	*mut-7*	Ø	✓
	cid-1	✓	✓
	ekl-1	✓	✓
	gfl-1	Ø	✓
	mes-2	✓	✓
	ekl-4	✓	✓
	mes-6	✓	✓
	rha-1	✓	✓
	ekl-6	Ø	✓
	zfp-1	✓	✓
	mut-2	✓	✓
	ekl-5	Ø	**Ø**
	mes-3	Ø	**Ø**
	mut-16	Ø	✓
	rde-2	Ø	**Ø**
Argonaute proteins	*alg-1*	✓	✓
	rde-1	✓	✓
	ppw-1	Ø	✓
	csr-1	Ø	✓
	ppw-2	Ø	✓
	sago-1	✓	✓
	alg-2	✓	✓

	Gene	*T. circumcincta*	*H. contortus*
	ergo-1	Ø	✓
	prg-1	Ø	✓
	nrde-3	Ø	**Ø**
	sago-2	Ø	✓
	prg-2	✓	✓
	C14B1.7[a]	Ø	✓
	wago-1	Ø	✓
	C04F12.1	✓	✓
	wago-4	✓	✓
	hrde-1	Ø	✓
	alg-3	✓	✓
	wago-10	Ø	✓
	wago-2	Ø	✓
	hpo-24	Ø	✓
	T23B3.2	Ø	✓
	wago-11	✓	✓
	wago-5	Ø	✓
	C06A1.4[a]	Ø	✓

Table 4.4. RNAi pathway genes in the genome of *T. circumcincta* after searching against the partially annotated *T. circumcincta* genome in Sanger database (https://www.sanger.ac.uk/cgi-bin/blast/submitblast/t_circumcincta). The accession number of the primary hit is mentioned and also the E-value and the percentage of coverage and the identities of a reciprocal tBLASTx search against the nucleotide selection of *C. elegans* in NCBI.

	Gene	*T. circumcincta*' accession number	Query cover %	Identities %	E value
siRNA synthesis proteins	*drh-3*	tc-219f06.q1k bases 35 to 773 (SL to QR)	23%	46%	2e-08
	drsh-1	Supercontig_0012 923	19%	60%	6e-17
	xpo-1	Supercontig_0010 793	4%	62%	9e-05
	xpo-2	Supercontig_0011 469	5%	64%	5e-04
	dcr-1	Supercontig_0020 298	44%	69%	3e-38
	pash-1	No hit	No hit	No hit	No hit

	Gene	*T. circumcincta*' accession number	Query cover %	Identities %	E value
	rde-4	No hit	No hit	No hit	No hit
	xpo-3	tc-558c18.p1k bases 32 to 823 (SL to QR)	38%	81%	3e-25
	drh-1	Supercontig_0019 946	26%	52%	3e-31
siRNA amplification proteins	*smg-2*	Supercontig_0000 746	62%	68%	1e-102
	smg-6	No hit	No hit	No hit	No hit
	ego-1	Supercontig_0020 277	11%	44%	8e-09
	rrf-3	tc-441o12.p1k bases 34 to 755 (SL to QR)	26%	76%	8e-13
	rrf-1	Supercontig_0004 603	10%	57%	9e-18
	smg-5	Supercontig_0012 434	4%	36%	4e-04
	rsd-2	No hit	No hit	No hit	No hit
RNAi signal spreading proteins	*rsd-3*	No hit	No hit	No hit	No hit
	sid-1	tc-305c16.p1k bases 202 to 705 (QL to QR)	27%	35%	1e-06
	rsd-6	No hit	No hit	No hit	No hit
	sid-2	No hit	No hit	No hit	No hit
RISC proteins	*tsn-1*	tc-26i11.p1k bases 31 to 753 (SL to QR)	88%	80%	2e-68
	vig-1	No hit	No hit	No hit	No hit
	ain-1[a]	tc-202j10.p1k bases 28 to 698 (SL to QR)	31%	56%	2e-09
	ain-2[a]	No hit	No hit	No hit	No hit
RNAi inhibitor proteins	*eri-1*	tc-330g13.q1k bases 26 to 781 (SL to QR)	28%	47%	4e-12
	xrn-2	tc-191h06.q1k bases 61 to 814 (QL to QR)	53%	76%	6e-56
	adr-2	tc-24a12.p1k bases 82 to 823 (QL to QR)	31%	63%	4e-15

	Gene	*T. circumcincta*' accession number	Query cover %	Identities %	E value
	xrn-1	Supercontig_0005 136	17%	77%	3e-95
	adr-1	tc-22m15.p1k bases 150 to 569 (QL to QR)	67%	51%	2e-16
	lin-15b	No hit	No hit	No hit	No hit
	eri-5	No hit	No hit	No hit	No hit
	eri-6/7	No hit	No hit	No hit	No hit
	eri-3	No hit	No hit	No hit	No hit
Nuclear RNAi effectors	*mut-7*	No hit	No hit	No hit	No hit
	cid-1	Supercontig_0011 254	16%	78%	6e-24
	ekl-1	tc-23d21.q1k bases 36 to 823 (SL to QR)	17%	58%	1e-06
	gfl-1	No hit	No hit	No hit	No hit
	mes-2	tc-333n08.p1k bases 34 to 785 (SL to QR)	41%	63%	1e-17
	ekl-4	Supercontig_0014 695	8%	45%	3e-09
	mes-6	tc-276j14.p1k bases 34 to 786 (QL to QR)	22%	54%	1e-08
	rha-1	tc-19m23.p1k bases 87 to 775 (QL to QR)	58%	67%	2e-36
	ekl-6	No hit	No hit	No hit	No hit
	zfp-1	Supercontig_0016 848	7%	76%	5e-38
	mut-2	Supercontig_0015 612	11%	50%	1e-07
	ekl-5	No hit	No hit	No hit	No hit
	mes-3	No hit	No hit	No hit	No hit
	mut-16	No hit	No hit	No hit	No hit
	rde-2	No hit	No hit	No hit	No hit
Argonaute proteins	*alg-1*	tc-361f07.p1k bases 99 to 680 (QL to QR)	90%	95%	1e-80
	rde-1	tc-294e04.q1k bases 30 to 818 (SL to QR)	21%	66%	3e-11

Gene	*T. circumcincta*' accession number	Query cover %	Identities %	E value
ppw-1	No hit	No hit	No hit	No hit
csr-1	No hit	No hit	No hit	No hit
ppw-2	No hit	No hit	No hit	No hit
sago-1	tc-186k21.q1k bases 38 to 747 (QL to QR)	34%	31%	5e-09
alg-2	tc-392c09.q1k bases 30 to 851 (SL to QR)	67%	85%	2e-73
ergo-1	No hit	No hit	No hit	No hit
prg-1	No hit	No hit	No hit	No hit
nrde-3	No hit	No hit	No hit	No hit
sago-2	No hit	No hit	No hit	No hit
prg-2	tc-219d05.p1k bases 53 to 832 (QL to QR)	47%	73%	2e-46
C14B1.7[a]	No hit	No hit	No hit	No hit
wago-1	No hit	No hit	No hit	No hit
C04F12.1	tc-306e08.p1k bases 145 to 455 (QL to QR)	60%	54%	4e-07
wago-4	tc-544p08.p1k bases 37 to 787 (SL to QR)	24%	67%	3e-20
hrde-1	No hit	No hit	No hit	No hit
alg-3	tc-86o21.q1k bases 36 to 782 (SL to QR)	23%	75%	4e-18
wago-10	No hit	No hit	No hit	No hit
wago-2	No hit	No hit	No hit	No hit
hpo-24	No hit	No hit	No hit	No hit
T23B3.2	No hit	No hit	No hit	No hit
wago-11	tc-453k04.p1k bases 33 to 888 (SL to QR)	16%	40%	8e-05
wago-5	No hit	No hit	No hit	No hit
C06A1.4[a]	No hit	No hit	No hit	No hit

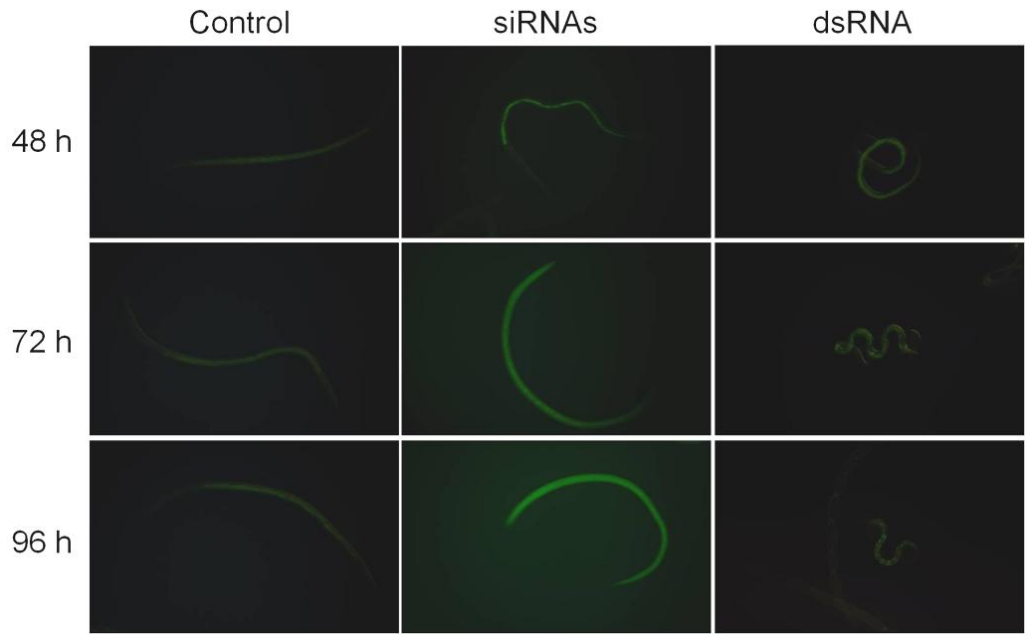


Figure 4.7. Uptake of fluorescently-labelled siRNA and dsRNA from CO_2-exsheathed L3 *T. circumcincta*. The columns (from left to right) represent xL3 soaked in EBSS (control), FITC-labelled siRNAs and FITC-labelled dsRNA, respectively. The rows (from top to bottom) represent the duration of soaking, i.e. 48, 72 and 96 hours. The uptake of both siRNA and dsRNA was indicated by increased fluorescence after 48 hours of soaking, with the siRNA being more intense than the dsRNA. Uptake of siRNA increased with time but this was not clearly evident with dsRNA.

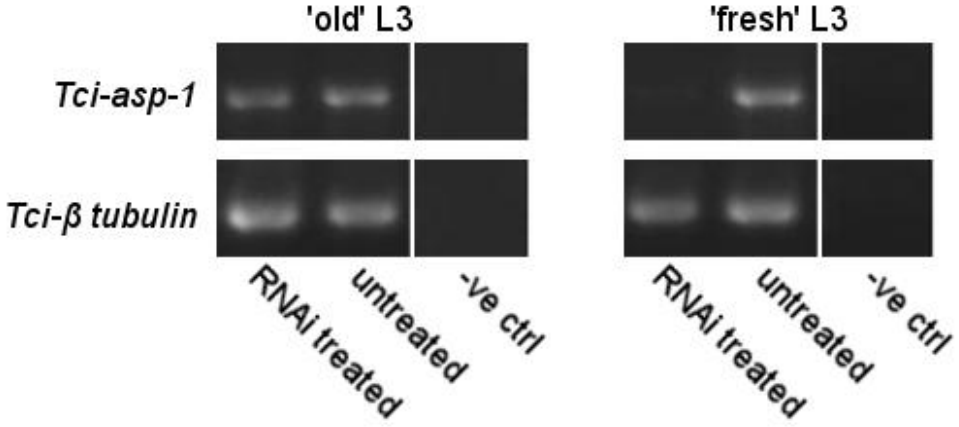


Figure 4.8. RT-PCR detection of *Tci-asp-1* transcript in CO_2-exsheathed L3 *T. circumcincta* stored for a year ('old' L3) and a week ('fresh' L3) after soaking in *Tci-asp-1*-specific dsRNA for 1 hour (RNAi treated) compared with untreated worms (untreated). RNAi was successful in the 'fresh' L3 (stored for a week at +4ºC) but not in the 'old' L3 (stored for a year at +4ºC) Negative no-template controls were included (-ve ctrl). *Tci-β tubulin* was used as a housekeeping gene.

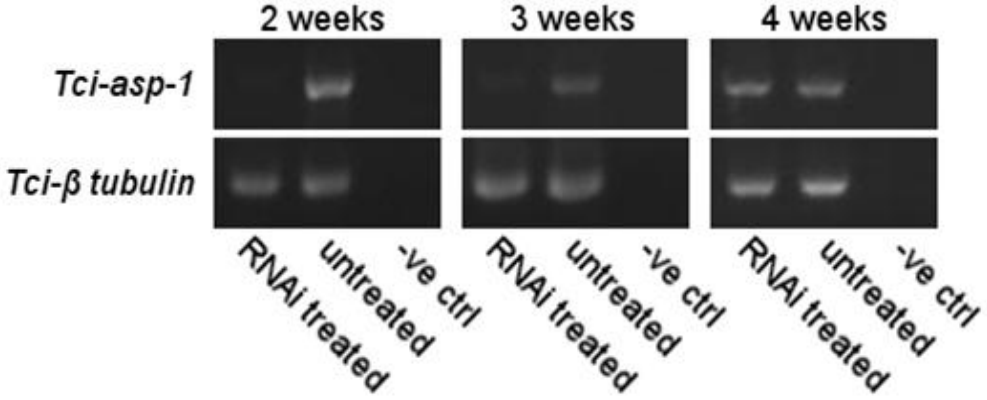


Figure 4.9. RT-PCR detection of *Tci-asp-1* transcript in CO_2-exsheathed L3 *T. circumcincta* after soaking in *Tci-asp-1*-specific dsRNA for 1 hour (RNAi treated) compared with untreated worms (untreated). The period that the L3 were susceptible to RNAi after the copro-culture was examined by using L3 stored at +4ºC for 2, 3 and 4 weeks. L3 were susceptible to RNAi for the first 3 weeks after the copro-culture, however they were not susceptible to RNAi after storing them for 4 weeks. Negative no-template controls were included (-ve ctrl). *Tci-β tubulin* was used as a housekeeping gene.

4.3.5 Effect of RNAi with hsiRNA in 'fresh' larvae

The effect of hsiRNA was tested on 'fresh' L3. The RNAi experiments with sL3 stored for less than a week showed a successful repeat of knockdown for *Tci-asp-1* after soaking for 1 hour in dsRNA and hsiRNA specific for *Tci-asp-1*, with the effect being more robust after soaking with the dsRNA (Figure 4. 10). However, RNAi was not successful for the additional two targets (*Tci-mif-1* and *Tci-saa-1*), with the dsRNA or the hsiRNA (Figure 4. 10).

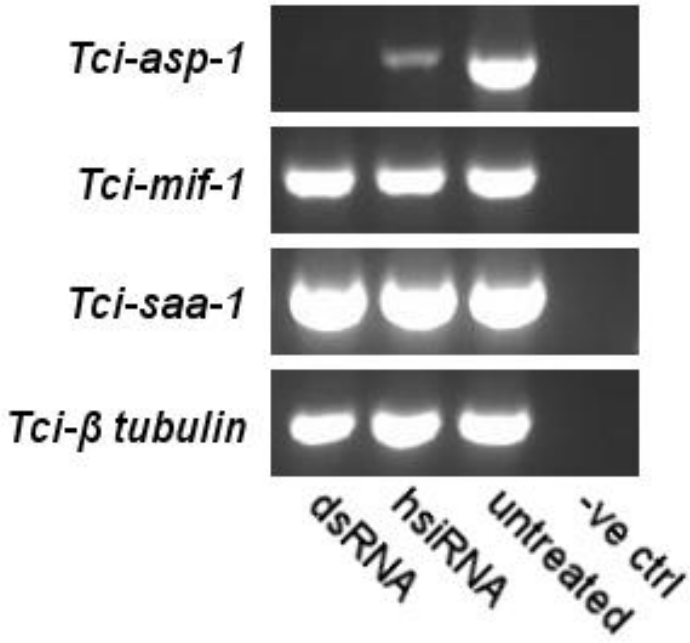


Figure 4.10. RT-PCR detection of the RNAi target genes (*Tci-asp-1*, *Tci-mif-1*, *Tci-saa-1*) transcripts in CO_2-xL3 *T. circumcincta* after soaking in gene-specific dsRNA and hsiRNA for 1 hour (dsRNA and hsiRNA, respectively) compared with untreated worms. The results have shown a successful silencing effect for *Tci-asp-1* but not for *Tci-mif-1* and *Tci-saa-1*. The effect of dsRNA was more robust compared to the hsiRNA. Negative no-template controls were included (-ve ctrl). *Tci-β tubulin* was used as a housekeeping gene.

4.4 Discussion

In this chapter, it has been demonstrated that RNAi was successful in *T. circumcincta* after as little as 1 hour of soaking in gene-specific dsRNA and that the transcript levels of *Tci-asp-1* recover 48 hours after the end of the 1-hour soak. Nevertheless, the silencing effect was not only gene-dependent, but also inconsistent after targeting the same genes in different batches of parasite larvae. It was also shown that half of the RNAi pathway genes that are considered to be 'essential' for successful RNAi were present in the *T. circumcincta* genome and that delivery of dsRNA to the parasites was successful. A close homologue of *C. elegans rde-4*, which was thought to be absent, was found in the genome of *H. contortus* (Geldhof *et al.*, 2006b, Dalzell *et al.*, 2011). Finally, very important findings here were: the silencing effect of RNAi appears to be highly dependent on the storage period of the L3 prior to use, with larvae stored for less than a month being susceptible to RNAi whilst 'older' larvae were not; and soaking 'fresh' larvae in gene-specific hsiRNA results in successful silencing of the gene. The latter, to my knowledge, was tried for the first time in *T. circumcincta* in this study.

The RNAi targets that were chosen for *T. circumcincta* included five ASP genes (*tdc00462*, *Tci-asp-1*, *tdc00691*, *tdc00879* and *tdc01479*), *Tci-mif-1* and *Tci-saa-1*. The selection of the ASP genes for RNAi here was based on the results of Chapter 3 (Figure 3.5) and previous studies (Nisbet *et al.*, 2010b, Nisbet *et al.*, 2013). Particularly, *Tci-asp-1* was chosen because not only is it transcribed in all the parasitic stages (xL3, L4, adults; Chapter 3, Figure 3.5) but also it is a target of the early immune response in previously infected sheep (Nisbet *et al.*, 2010b) as well as being a component of the recombinant vaccine that has been used with success to immunise sheep against *T. circumcincta* challenge (Nisbet *et al.*, 2013). *Tdc00462* and *tdc00879* were chosen because they are also transcribed in the parasitic stages (Chapter 3, Figure 3.5). The remaining ASP genes (*tdc00691* and *tdc01479*) were chosen because they are transcribed in all the stages (Chapter 3, Figure 3.5) and thus represent genes that are also transcribed in stages prior to exsheathment. *Tci-mif-1* and *Tci-saa-1* were chosen because they also are components of the recombinant vaccine (Nisbet *et al.*, 2013) and are known to be transcribed in the L3 stage (Zhan *et*

al., 2004, Nisbet *et al.*, 2009, Nisbet *et al.*, 2010a). *Tci-mif-1* is a potentially immunosuppressive molecule (Nisbet *et al.*, 2010a) and *Tci-saa-1* is an immunogenic homologue protein of a protective antigen from *Ancylostoma caninum* (Zhan *et al.*, 2004, Nisbet *et al.*, 2009).

The RNAi experiments in *T. circumcincta* initially indicated successful silencing for the majority of the ASP target genes, i.e. *tdc00462*, *Tci-asp-1*, *tdc00691* and *tdc01479*, after soaking 10,000 xL3 in gene-specific dsRNA for 1 hour. However, the RNAi was not successful for the rest of the target genes, i.e. *tdc00879*, *Tci-mif-1*, *Tci-saa-1*. These results highlight the inconsistent nature of RNAi between different genes, which has been reported in the past for several parasitic nematode species (Britton and Murray, 2006, Geldhof *et al.*, 2007). Since RNAi was successful only for some of the ASP genes, further experiments focussed on *Tci-asp-1* because it is transcribed in the stages of the parasite that inhabit the host.

In previous studies, the protein and the transcript of *Tci-asp-1* were identified in the L4 stage of *T. circumcincta*, but not in sodium hypochlorite exsheathed L3 (Nisbet *et al.*, 2010b). It was demonstrated in the previous Chapter that the transcript of *Tci-asp-1* could be identified in the xL3 stage after CO_2 exsheathment. Here, detection of Tci-ASP-1 protein was investigated. The CO_2-xL3 were *in vitro* cultured for 96 hours and RNA and protein preparations produced after 24, 48, 72 and 96 hours from initiation of the culture. The results indicated that the transcript levels of *Tci-asp-1* increase with time with the highest levels observed four days after exposure to CO_2. The ASP-1 protein could not be identified in extracts obtained at any of the time points. This implies that *Tci-asp-1* is transcribed but not translated in the xL3. Potential explanations for that might be: that a trigger from the host is required to initiate the translation of the gene; or that the protein quantity is too low to be revealed. The latter possibility could be tested by employing a more sensitive detection method such as ECL detection.

It is known that RNAi does not have a permanent effect (Domeier *et al.*, 2000). The duration of silencing until the transcript of the target gene recovers to normal levels after the dsRNA exposure depends on several *smg* (suppressor of

morphological effects on genitalia) genes (Domeier *et al.*, 2000). The transcript recovery experiments performed here showed that, after 1 hour of soaking in *Tci-asp-1* specific dsRNA, the silencing effect was still obvious 24 hours after removing the dsRNA, but after 48 hours the transcript had returned to similar levels compared to those in the untreated control worms. After initial successes with RNAi knockdown here, the results became inconsistent and the silencing effect could not be reproduced in subsequent experiments. The inconsistencies coincided with the replacement of the stock L3 that were used, which indicated that the variability of RNAi results in the same target-gene could be due to different worm batches.

In the literature, there have been several suggestions to explain the reason for variability in RNAi in parasitic nematodes. In particular, it has been suggested that inconsistencies could be a consequence of inappropriate dsRNA delivery methods (Viney and Thompson, 2008); problematic culture conditions of the parasitic nematodes that do not allow them to continue development (Knox *et al.*, 2007); non-specific effects of the by-products (i.e. partially degraded dsRNA, anti-sense RNA or siRNAs) during the synthesis of dsRNA (Knox *et al.*, 2007); or the absence of 'essential' RNAi pathway genes (Viney and Thompson, 2008). In addition, it has been demonstrated that consistency of the silencing effect may depend on the site of the expression of the gene (Samarasinghe *et al.*, 2011). Samarasinghe *et al.* (2011) suggested that the expression location plays an important role in RNAi because genes expressed in sites accessible to the external environment (i.e. the intestine, excretory or amphid cells) appeared to be more susceptible to RNAi in *H. contortus* than genes expressed at other sites. To investigate potential reasons for the inconsistencies in RNAi observed here, a bioinformatics search of the *T. circumcincta* genome was performed to determine the presence or absence of the RNAi pathway genes considered to be 'essential' for successful RNAi (Dalzell *et al.*, 2011). The bioinformatics analysis showed that 37 out of 74 'essential' RNAi pathway genes were present in the existing databases (Tables 4.3 and 4. 4). One explanation for this could be that the genome coverage available was not complete. Thus, because there is greater coverage for the genome of another Clade V nematode, *H. contortus*, this genome was searched not only for the genes that were

not identified in the *T. circumcincta* genome, but also for the RNAi pathway genes that were not identified in the *H. contortus* genome, in previous studies (Dalzell *et al.*, 2011). The search showed that 66 out of 74 'essential' genes were present in *H. contortus* (Table 4. 3). These included 19 that were not identified before by Dalzell *et al.* (2011). A very interesting finding was the identification of *rde-4*, which was previously thought to be absent in *H. contortus* (Geldhof *et al.*, 2006b, Dalzell *et al.*, 2011) and was thought to be a possible explanation for the failure of RNAi observed previously with several *H. contortus* genes (Geldhof *et al*, 2006; Knox, *et al*, 2007). The identification of the additional genes was achieved probably because *H. contortus* genome was not fully annotated until recently (Laing *et al.*, 2013). The two bioinformatics searches and the fact that the RNAi pathway was activated before for some of the ASP targets suggested that the genes required for a successful RNAi, and a functional RNAi pathway, are present in *T. circumcincta*.

Another potential reason for the observed inconsistencies is inappropriate dsRNA delivery methods (Viney and Thompson, 2008). The delivery of dsRNA was examined here by soaking xL3 in FITC-labelled siRNAs and dsRNA. The experiments showed that siRNA and dsRNA delivery was successful after a soaking period of 48 hours or greater. The untreated larvae displayed a level of auto-fluorescence and the uptake of the FITC-labelled siRNAs and dsRNA was not clear after 1 and 24 hours of soaking. The intensity of the fluorescence with the siRNAs was much greater compared with that arising following soaking in dsRNA. Nonetheless, it cannot be suggested that the siRNA delivery was better than the dsRNA delivery. Even though the concentration of both the FITC-labelled siRNAs and dsRNA was the same (1 mg/ml), the number of the individually FITC-labelled siRNAs was approximately 10-fold higher than the individually FITC-labelled dsRNAs. Since the RNAi experiments were unsuccessful, an alternative protocol was examined. xL3 were soaked in hsiRNAs because it had been proven previously to be successful in the filarial nematode, *B. malayi* (Landmann *et al.*, 2012). The results of these specific experiments showed that soaking xL3 in *Tci-asp-1*-specific hsiRNA for 1 hour did not result in knock down.

As mentioned above, the silencing effect could not be reproduced after the replacement of the original stock L3. Thus, inconsistencies in knockdown could be more related to the condition of the larvae at the start of experiment, rather than the soaking conditions themselves. As a result, storage period of the larvae before use in the experiments was examined as a factor that could affect RNAi repeatability. An experiment was conducted to compare susceptibility of L3 stored for a year and L3 stored for a week after culture. The soakings here took place in parallel (same conditions and dsRNA) and the only difference was the larvae that were used. The results indicated successful RNAi in L3 stored for a week but not in L3 stored for a year. The time-course experiment showed that the L3 were susceptible to RNAi for up to 3 weeks after recovery from copro-culture. After the results that showed that the outcome of RNAi experiments in *T. circumcincta* is associated with L3 storage time, 'fresh' L3 (stored for a week) were soaked in dsRNA and hsiRNA targeting *Tci-asp-1*, *Tci-mif-1* and *Tci-saa-1*. There was successful repeat of knockdown for *Tci-asp-1* after soaking in both dsRNA and hsiRNA, and the effect was better after soaking with the dsRNA. The results also showed that RNAi was not successful for the additional two targets. A search in the literature revealed a study in the flour beetle, *Tribolium castaneum*, where the efficacy of dsRNA and siRNA was compared (Wang *et al.*, 2013). Their results showed that siRNAs were less effective than their corresponding dsRNA, potentially due to fast degradation of the siRNAs (Wang *et al.*, 2013). Nevertheless, there have not been similar studies conducted in helminths or other endoparasites.

In conclusion, RNAi was successful, but gene specific, after soaking larvae in gene-specific dsRNA for one hour. The transcript of *Tci-asp-1* fully recovered to pre-soaking normal levels, in the absence of dsRNA, 48 hours after the end of the soak period. Inconsistencies were observed after replacement of stock L3, and thus, storage period was found to be an important factor in RNAi repeatability in *T. circumcincta*, with larvae stored at 4°C for up to 3 weeks being susceptible to RNAi whilst 'older' larvae were not.

Chapter 5: Characterization of exosomes in *T. circumcincta*

5.1 Introduction

There are several organelles present within cells that comprise molecules that have a specific cellular function (Andersen and Mann, 2006). There are also organelles (extracellular microvesicles; EMVs) that are released by the cell into the extracellular microenvironment. Such organelles include exosomes, shedding microvesicles and apoptotic bodies (Mathivanan *et al.*, 2010). As mentioned in Chapter 1, these three types of organelles differ primarily in size and how they are generated. Particularly, exosomes are small membranous vesicles of endocytic origin, approximately 50 – 100 nm in diameter (Thery *et al.*, 2006), and were initially described in the 1980s (Pan and Johnstone, 1983). Exosomes originate from large multivesicular bodies (MVBs) and are released to the extracellular space after fusion of the MVBs with the plasma membrane (van Niel *et al.*, 2006, Simpson *et al.*, 2009). Shedding microvesicles are larger (100 - 1000 nm) and are formed by direct blebbing of the plasma membrane (Cocucci *et al.*, 2009). Apoptotic bodies are larger membrane vesicles (1-4 μm) that are formed during apoptosis (Hristov *et al.*, 2004). Although the exosomes were initially characterized over 30 years ago, their precise nature and function have only been tried to be elucidated over the last decade and has been reviewed by many (Couzin, 2005, Simons and Raposo, 2009, Mathivanan *et al.*, 2010, Bobrie *et al.*, 2011, Thery, 2011, Montaner *et al.*, 2014). The growth in this field of research is due to the discovery of the important roles that exosomes may play in intercellular signalling and cell-cell communication.

Although EMVs were initially thought to be cell debris or indicators of cell death (Hristov *et al.*, 2004), two major breakthroughs suggested that exosomes are specific functional cell products rather than mere debris. The proteomic analyses of exosomes released from different cell types revealed that these vesicles contain proteins that belong to a few specific families and are common in the exosomes from different cell types (Thery *et al.*, 1999, Thery *et al.*, 2001, Mathivanan and Simpson, 2009, Mathivanan *et al.*, 2012, Simpson *et al.*, 2012). The second breakthrough was

the discovery that exosomes also contain mRNA and miRNA (microRNA), and that some of the mRNAs could be translated in target cells (Valadi *et al.*, 2007). The initial studies that indicated the importance of exosomes were from the field of immunology, where it was demonstrated that not only B-lymphocytes (Raposo *et al.*, 1996), but also dendritic cells (Zitvogel *et al.*, 1998), secrete exosomes which contain molecules that affect immune responses (Andreola *et al.*, 2002, Thery *et al.*, 2002). The results of these studies suggested a potential role of exosomes in intercellular communication in the immune system. Other potential functions, in addition to the activation and/or suppression of the immunological responses, include: the transfer of pathogenic proteins, tissue repair and neural communication (Couzin, 2005, Simons and Raposo, 2009).

Research regarding exosomes has been expanded to other organisms including parasitic helminths (cestodes, trematodes and nematodes) and protozoa (Montaner *et al.*, 2014). The first report of EMVs in parasites was in the 1960s when they were described as "small membrane-limited vesicles" during the characterization of the tegument of the liver fluke, *Fasciola hepatica* (Threadgold, 1963). Another study in the 1980s described the presence of "membrane bound vesicles" during the characterization of the surface antigens of the trematode, *Echinostoma caproni* (Andresen *et al.*, 1989). A recent study has shown that the same trematode parasites (*F. hepatica* and *E. caproni*) actively release exosomes into their micro-environment (Marcilla *et al.*, 2012). Other examples of organisms that were found to release exosomes include, among others: the nematodes, *Caenorhabditis elegans* (Liegeois *et al.*, 2006) and *Heligmosomoides polygyrus* (Buck *et al.*, 2014); the cestode *Echinococcus multilocularis* (Eger *et al.*, 2003); and the protozoan parasites *Leishmania donovani* (Silverman *et al.*, 2010) and *Toxoplasma gondii* (Pope and Lasser, 2013). Marcilla *et al.* (2012) showed that intestinal rat cells actively take up exosomes secreted by *F. hepatica* and *E. caproni*. Moreover, Silverman *et al.* (2010) suggested that exosomes released by *L. donovani* are predominately immunosuppressive and can modulate innate and adaptive immune responses of the host by affecting monocyte and dendritic cell function. A recent study demonstrated that small RNAs contained in exosomes secreted by *H. polygyrus* regulate genes of

the innate immune system (Buck *et al.*, 2014). These observations support the belief that exosomes play an important role in host-parasite communication. Furthermore, based on these observations, it has been suggested that exosomes could be potential candidates for vaccines and/or targets for pharmaceutical intervention (Marcilla *et al.*, 2012).

The work here examined whether exosomes could be found in the excretory/secretory (ES) products of *T. circumcincta*. To date, there have been no reports on whether parasitic nematodes that infect ruminants release exosomes. Furthermore, a detailed proteomics analysis was conducted to characterize the contents of the exosomes. Finally, an important consideration was to examine whether the exosomes are recognized by the host immune response. As a result, immunoblots were performed to examine the immunogenicity of *T. circumcincta* exosome contents in sheep administered with experimental infections of the parasite.

5.2 Materials and methods

5.2.1 Parasite material

Exosomes were prepared from the ES products of fourth stage larvae (L4) of *T. circumcincta*. The L4 were harvested following methods described in previous studies (Knox and Jones, 1990). In particular, five, helminth-free lambs were infected, each with approximately 150,000 sheathed *T. circumcincta* L3 (strain MTci2; an anthelmintic-susceptible laboratory isolate from Moredun Research Institute). Seven days later, the lambs were euthanized to retrieve L4 stages. The abomasum was removed and processed individually. The L4 were retrieved from the mucosa and from the abomasal contents. Each abomasum was opened along the greater curvature, the contents emptied into a container and the luminal side washed in a container of warm physiological saline (37°C) to remove remaining food debris. To retrieve mucosal L4, the abomasum was pinned to a polystyrene float with its luminal side facing up. The abomasum was left to float in a large funnel containing warm physiological saline (37°C; with the folds of the abomasum hanging down in the funnel). The funnel with the floating abomasum was incubated in a hot room

(37°C) for 2 h to allow L4 to emerge from the mucosa and sink into the funnel. Following that, the bottom portion of saline was carefully tapped off into a measuring cylinder. The funnel was topped up with physiological saline to ensure the abomasum still floated and incubated for a further 2 h in the hot room. Again, the bottom portion of saline was carefully tapped off into a measuring cylinder. The mucosal L4 from the five donor lambs were pooled together and allowed to settle. The abomasal contents were transferred in a piece of muslin, which was tied with a cable tie to form a pouch to retrieve luminal L4. The pouch containing the abomasal contents was left to float in a large funnel containing warm physiological saline (37°C) to allow the L4 to migrate through the muslin. The funnel was incubated at 37°C for two periods of 2 h each and the worms collected at the end of each incubation period. Finally, the luminal L4 from the five donor lambs were pooled together and allowed to settle. The luminal and mucosal L4 were pooled and the physiological saline containing the larvae divided into 50 ml falcon tubes (Cellstar® tubes, Greiner bio-one). The tubes were centrifuged at 50 *g* (no brake) at room temperature for 5 min to aid pelleting, the supernatant aspirated and the larval pellets pooled together. When all larvae were gathered in a single tube, they were centrifuged again and the supernatant discarded. The larval pellet was washed with 20 ml 1xPBS and incubated at 37°C for 20 min (the tube was inverted every 5-10 min). The L4 were centrifuged again (50 *g* at room temperature for 5 min, no brake) and the PBS wash repeated three times in total. After the final centrifugation, L4 were given a final wash using nematode culture medium (RPMI; Gibco®, Life technologies™). The worms were centrifuged, the supernatant removed and the L4 re-suspended in 40 ml nematode culture medium. Finally, they were incubated overnight in a cell culture flask with a vent cap (75 cm^2 Cell culture flask, Corning) at 37°C with 5% CO_2. The RPMI was prepared aseptically as follows: RPMI 1640 (500ml) was supplemented with sterile L- glutamine (10ml of 100mM), D-glucose (50ml; 5g in 50ml), penicillin/streptomycin (5ml of 10,000µg/ml), amphotericin B (62.5 mg), gentamycin sulphate (12.5mg) and Hepes solution (10ml; 1M Sigma-Aldrich).

5.2.2 Harvesting of *T. circumcincta* L4 ES products

The collection of the ES material took place in a sterile hood. The culture media with the L4 were transferred to a 50 ml falcon tube (Cellstar® tubes, Greiner bio-one) and centrifuged at 50 *g* at room temperature for 5 min. The supernatant (containing the 24 h ES products) was aspirated and transferred to a fresh 50 ml falcon tube. The L4 were re-suspended in fresh nematode culture medium (RPMI, 40ml), returned to the culture flask and incubated for another 24 h at 37°C with 5% CO_2. The ES products were centrifuged briefly (50 *g* at room temperature for 5 min) to pellet any worms that were accidentally aspirated and the supernatant filtered using a 0.22 µm syringe filter (millex® GP). The filtered ES products were divided into two equal aliquots (one to be processed for exosomes purification and the other for total ES preparation) and frozen at -80°C until the day they would be processed. The same protocol was followed the following two days for harvesting of 48 h and 72 h ES products.

5.2.3 Exosomes purification

The same protocol as in previous publications was followed (Buck *et al.*, 2014). The ES samples, from 24, 48 and 72 h, were thawed at room temperature. A Beckman ultra-centrifuge with the SW40 swing out rotor (cooled overnight at 4°C) and Beckman polyallomer 14 ml centrifuge tubes (Beckman Coulter) were used. The tubes were filled with at least 12.5 ml of sample, weighed on a fine balance and brought to the same weight to the second decimal of each other. The samples were centrifuged at 100,000 *g* for 2 h at 4°C. The exosome-free supernatant was gently aspirated and transferred to a different 50 ml falcon tube (Cellstar® tubes, Greiner bio-one). The polyallomer tubes were re-filled with the remaining sample, weighted on the fine balance as before and centrifuged at 100,000 *g* for 2 h at 4°C. Again, the supernatant was carefully transferred in a different falcon tube and stored at -80°C. The exosome pellets in the polyallomer tubes were washed twice with at least 12.5 ml PBS. After each wash, the tubes were centrifuged at 100,000 *g* for 2 h at 4°C and the supernatant carefully aspirated and discarded. Before the final wash, all pellets

were re-suspended (by pipetting and vortexing) and pooled together in a single tube. Finally, the supernatant of the sample was carefully removed leaving approximately 100-200 µl of PBS to re-suspend the exosome-containing pellet. The protein concentration was measured with Qubit® 2.0 Fluorometer (Life technologies™) and the purified exosomes stored at -80°C.

5.2.4 Concentration of L4 total and exosome-free ES products

The samples with the total ES products (Section 5.2.2) and with the exosome-free ES products (Section 5.2.3) were thawed at room temperature. Amicon Ultra-15 centrifugal filter units (MWCO 10 kDa; Sigma Aldrich) were used to concentrate the total and the exosome-free ES products individually. The units were filled and centrifuged at 3,000 *g* for 20 min at 4°C. The filtrate was discarded and the units were re-filled with sample and centrifuged again under the same conditions. Finally, the units were washed twice with pre-chilled PBS to buffer exchange the ES products. After each wash, the units were centrifuged at 3,000 *g* for 20 min at 4°C. The concentration of the protein in these samples was measured using the Pierce® BCA Protein Assay kit (Thermo scientific) based on the manufacturer's protocol.

5.2.5 Transmission Electron Microscopy (TEM)

An aliquot (8 µl) of the exosomes preparation was fixed with an equal volume of paraformaldehyde 4% (Fisher scientific). The sample was given to the Electron Microscopy (EM) unit (Daniel Rutherford Building, The University of Edinburgh, King's Buildings). The technicians of the EM unit prepared the sample for the TEM as described in previous publications (Thery *et al.*, 2006). Then, the sample was scanned by TEM to confirm the presence or absence of exosomes. The exosome-free samples were not subjected to TEM.

5.2.6 Protein profiles of the exosomes, the exosome-free and total ES products

In order to examine the protein profile of the exosomes and compare it with the profiles of the exosome-free and total ES products, 2 μg of each sample were used. The samples were denatured at 70°C for 10 min after adding 2.5μl NuPAGE® LDS sample buffer (4X; Life Technologies™) and 1μl 10X NuPAGE® sample reducing agent (Life Technologies™). Following the denaturing step, 2 μg of each sample (10μl total volume each sample) were electrophoresed on NuPAGE® Novex® Bis-Tris 4–12% mini gels (Life Technologies™) under reducing conditions using NuPAGE® MES SDS running buffer (Life Technologies™). The proteins were stained with SimplyBlue™ SafeStain (Life Technologies™) based on the manufacturer's instructions. Another NuPAGE® gel was prepared as above and the proteins were stained with SilverQuest™ Silver Staining Kit (Life Technologies™) according to the manufacturer's instructions.

5.2.7 Proteomic analysis of the exosomes of L4 *T. circumcincta* 1-week post infection

Preparations that were confirmed to contain exosomes by TEM were subjected to proteomic analysis. Approximately 10 μg exosomes and 10μg exosome-free ES products were prepared, electrophoresed on NuPAGE® Novex® Bis-Tris 4–12% mini gels (Life Technologies™) and stained with SimplyBlue™ SafeStain (Life Technologies™) as described in Section 5.2.6. The NuPAGE® gel was given to the Proteomics facility of Moredun Research Institute for 'Sawn-Off Shotgun Protein Analysis' (SOSPA) as described in previous publications (Smith *et al.*, 2009).

The procedure was as follows: each of the two, SimplyBlue-stained gel lanes was excised then sliced horizontally from top to bottom to yield a series of 24 equal gel slices of 2.5 mm deep. Each of the resulting slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsinolysis procedures (Shevchenko *et al.*, 1996). Digests were transferred to HPLC sample vials and stored at 4^{o}C until required for liquid chromatography-electrospray ionisation-tandem mass

spectrometry (LC-ESI-MS/MS) analysis. Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon™ chromatography software (Dionex: http://www1.dionex.com). A micro-pump flow rate of 246 µl/min^{-1} was used in combination with a cap-flow splitter cartridge, affording a $^{1}/_{82}$ flow split and a final flow rate of 3 µl/min^{-1} through a 5 cm x 200 µm ID monolithic reversed phase column (Dionex-LC Packings) maintained at 50°C. Samples of 4 µl were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8-45% solvent B (80% acetonitrile, 0.1% ($^{v}/_{v}$) formic acid) and directed through a 3 nl UV detector flow cell. The LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (amaZon-ETD, Bruker Daltonics) via a low-volume (50µl/min^{-1} maximum) stainless steel nebuliser (Agilent, cat. no.G1946-20260) and ESI. Parameters for tandem MS analysis were based on those described previously (Batycka *et al.*, 2006). Deconvoluted MS/MS data in .mgf (Mascot Generic Format) were imported into ProteinScape™ V3.1 (Bruker Daltonics) proteomics data analysis software for downstream database mining of both Nembase4 (http://www.nematodes.org/nembase4/) and a cognate partial *T. circumcincta* genomic sequence (Moredun Research Institute) utilising the Mascot™ V2.3 (Matrix Science) search algorithm. The protein content of each individual gel slice was established using the "Protein Search" feature of ProteinScape™, whilst separate compilation of the proteins contained in all 24 gel slices for each sample were produced using the "Protein Extractor" feature of the software. Mascot search parameters were set in accordance with published guidelines (Taylor and Goodlett, 2005) and to this end, fixed (carbamidomethyl "C") and variable (oxidation "M" and deamidation "N,Q") modifications were selected along with peptide (MS) and secondary fragmentation (MS/MS) tolerance values of 0.5Da whilst allowing for a single 13C isotope.

The dataset assembled from the procedure described above was inspected manually using the MOlecular Weight SEarch (MOWSE) scores attained for

individual protein identifications. These were considered significant only if: a) two peptides were matched for each protein, *and* b) each matched peptide contained an unbroken "*b*" or "*y*" ion series represented by a minimum of four contiguous amino acid residues (Smith *et al.*, 2009). The results in ProteinScape™ V3.1 (Bruker Daltonics) from the cognate partial *T. circumcincta* genomic sequence included the closest homologue protein after a BLAST search, whilst the results from the Nembase4 search included the sequence ID of the protein. Each protein sequence from Nembase4 that fulfilled the above conditions was subjected to a manual Blast procedure (tblastn) against the full nucleotide collection to find the closest homologue protein. Finally, the proteins detected in the exosomes sample were searched in the ExoCarta database, which "provides with the contents that were identified in exosomes in multiple organisms" (http://www.exocarta.org/; Mathivanan and Simpson, 2009, Mathivanan *et al.*, 2012), and in previous publications (Marcilla *et al.*, 2012) to examine whether they were detected previously as exosomes related proteins. Moreover, they were searched against the results of previous studies that examined the gene expression and protein profile of total ES products derived from L4 *T. circumcincta* (Nisbet *et al.*, 2008, Smith *et al.*, 2009).

5.2.8 Immunogenicity of the exosomes

Immunoblots were performed to test whether serum IgG and IgA from sheep, which had been experimentally trickle infected for previous experiments at Moredun Research Institute (kindly donated by Dr Nisbet), recognized the proteins contained in the exosomes. Sheep were infected for 6 weeks (2,000 L3, three times per week) and had developed immunity against *T. circumcincta*. Serum from the same sheep before infection ('pre-immune') was used as a negative control. The immunoblot experiments were conducted as described in Chapter 4 (Section 4.2.4.). Initially, optimization experiments took place where different protein concentrations and dilutions of sera were examined using total ES products. This step showed that 5 μg of protein and a serum dilution of 1:200 (primary antibody) gave the best results. The

protocol for the immunoblot for antigen recognition by IgG was the following: 5 µg of each sample (exosomes, exosome-free and total ES products) were prepared and electrophoresed on NuPAGE® Novex® Bis-Tris 4–12% mini gels (Life Technologies™) under reducing conditions employing NuPAGE® MES SDS running buffer (Life Technologies™) as described in Section 5.2.6. The proteins were transferred onto Nitrocellulose Pre-Cut Blotting Membranes (0.2 µm pore size; Life Technologies™); the membranes washed briefly in TNTT (10 mM Tris, 0.5M NaCl, 0·05% Tween 20, 0.01% thiomersal, pH 7.4) and incubated in TNTT overnight at 4°C to block any non-specific protein adsorption. The blots were incubated with infected sheep sera, diluted 1:200 in TNTT, for 1 h. Negative controls were included by incubating blots with pre-immune sera, diluted 1:200 in TNTT, for the same period. Then, the membranes were washed three times in TNTT (10 min/wash); incubated for 1 h in donkey anti-sheep/goat IgG horseradish peroxidase (HRP) conjugate (AbD Serotec) diluted 1:1000 in TNTT; and washed three times in TNTT (10min/wash). Peroxidase activity was revealed using 3,3′-Diaminobenzidine (DAB) as substrate. The same protocol was used for the IgA studies. The only differences were the use of alternative secondary and tertiary antibody reagents. Particularly, after incubation with the sheep sera (immune and pre-immune), the membranes were washed three times in TNTT (10 min/wash); then incubated for 1 h in mouse anti-bovine/ovine IgA (AbD Serotec) diluted 1:1000 in TNTT; and washed again three times in TNTT (10 min/wash). Subsequently, they were incubated for 1 h in polyclonal rabbit anti-mouse immunoglobulins/HRP (Dako) diluted 1:1000 in TNTT; and washed again three times in TNTT (10min/wash). Peroxidase activity was revealed using the Amersham ECL Western Blotting system (GE healthcare Life sciences) following the manufacturer's protocol. All incubations and washes for the immunoblots were performed at room temperature with constant rocking.

5.3 Results

5.3.1 Exosomes in *T. circumcincta*'s L4 ES products

The TEM confirmed the presence of exosomes in ES products from *T. circumcincta* L4 stages collected from sheep at 1 week post infection, these being visible as small cup-shaped membrane vesicles (arrowed; Figure 5. 1, panels A and B). The protein profiles obtained for the different fractions (Figure 5. 2) showed that the profile for exosomes was different from the other two comparators (exosome-free and total ES products). Confirmation of these differences was sought using proteomics analysis (Section 5.3.2).

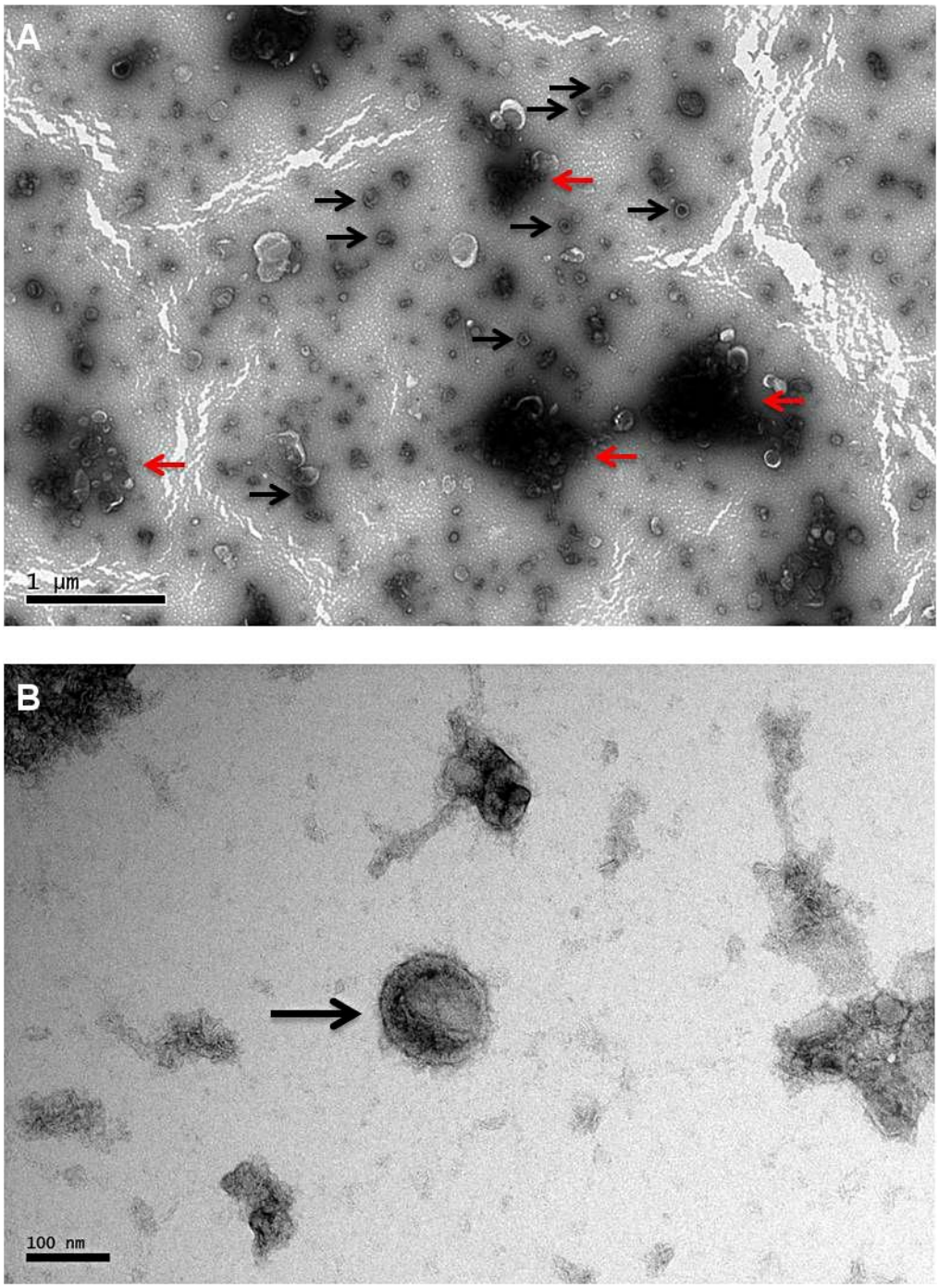


Figure 5.1. TEM confirmed the presence of exosomes in the ES products of *T. circumcincta* L4 stage. Panel A shows exosomes spread across the optical field (examples indicated with black arrows). Exosomes tend to aggregate which can be seen as the darker areas in panel A (examples indicated with red arrows). Panel B shows one exosome in higher magnification (black arrow).

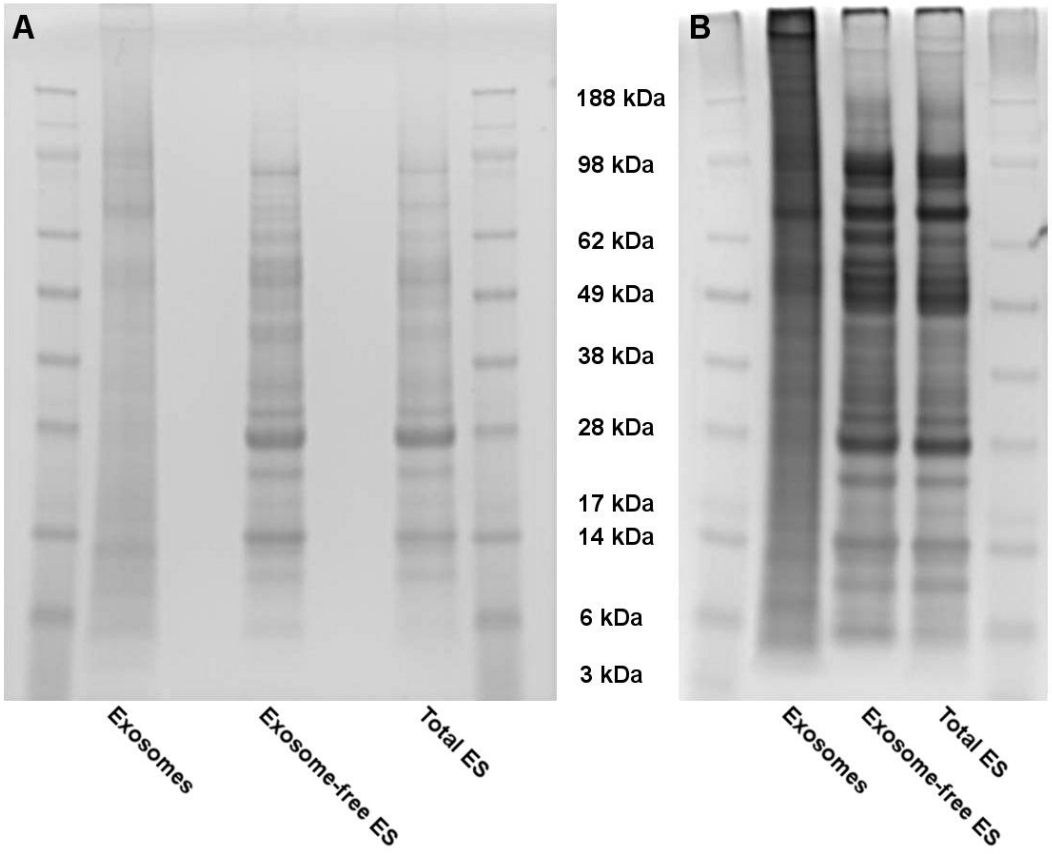


Figure 5.2. Protein profiles of exosomes, exosome- free and total ES products of *T. circumcincta* L4 stage. 2 µg of each sample (10 µl total volume) were subjected to electrophoresis under reducing conditions in duplicate. Subsequently, the proteins were stained with SimplyBlue™ SafeStain (Life Technologies™; Panel A) and with SilverQuest™ Silver Staining Kit (Life Technologies™). Exosomes had, apparently, quite a distinct visual profile compared to exosome-free and total ES products where the profiles were very similar.

5.3.2 Proteomic analysis of the exosomes and exosome-free ES products of L4 *T. circumcincta* 1-week post infection

Manual inspection of the proteomics dataset revealed 82 proteins in the exosomes preparation and 132 proteins in the exosome-free ES products. A total of 23 proteins were common in the two samples and the rest were unique in each sample. Since the aim of this Chapter was to characterize the exosomes released by L4 *T. circumcincta*, the subsequent analysis focussed on proteins identified in the exosome-enriched sample and whether these have been identified in exosomes of

other organisms. The proteins identified in the exosome-enriched and the exosome-free fractions and their closest homologues are shown in Appendices 1 and 2, respectively. The proteins of the exosome-enriched fraction were grouped according to broad function and these groups are shown in Table 5. 1 and Figure 5. 3. The protein groups were as follows: structural proteins (20.7% of the proteins); metabolic proteins (2.4%); nuclear proteins (4.9%); ASPs (12.2%); proteolytic enzymes (8.6%); 'ES' proteins (Excretory/Secretory proteins; 9.8%); cell to cell, cell to matrix interaction proteins (2.4%); ribosomal proteins (1.2%); Rab GTPases (6.1%); other function proteins (proteins that could not be classified in the previous categories; 11%); and proteins for which no homologues could be identified (20.7%).

Table 5.1. Broad function of the protein groups identified in the exosome-enriched samples.

*** indicates the identification of genes belonging to the same groups by Nisbet *et al*., 2008.**
‡ indicates the identification of genes belonging to the same groups by Smith *et al*., 2009.
† indicates the identification of the proteins in exosomes derived by other organisms (ExoCarta database and Marcilla *et al.*, 2012).

Protein	Number of proteins
1. Structural proteins	
Actin *†	13
WH2 actin-binding	1
Beta-tubulin†	2
Keratin *†	1
2. Metabolic Proteins	
E1-E2 ATPases †	1
Thioredoxin peroxidase *†	1
3. Nuclear proteins	
Histones †	3
Translation elongation factors †	1
4. Activation-associated secreted proteins	

Protein	Number of proteins
ASPs *‡	10
5. Proteolytic enzymes	
Cathepsin F *‡†	1
Aspartic protease†	3
Metalloproteases *‡†	1
Metallopeptidases *†	2
6. Excretory / Secretory proteins	
Excretory/secretory proteins *	8
7. Cell to cell, Cell to matrix interactions	
Clc-like (integral membrane component of tight junctions) *†	1
Transmembrane protein *†	1
8. Ribosomal proteins	
60S ribosomal protein *†	1
9. Rab GTPases	
Small GTPase Rab family †	5
10. Other function	
ADP-ribosylation factor †	3
Transthyretin-like protein *†	1
Saposin B *	2
Deoxynucleoside kinase	1
DB module family protein	1
Trp-Asp (WD) repeats circular profile protein †	1
11. No homology	
No homology proteins *‡†	17

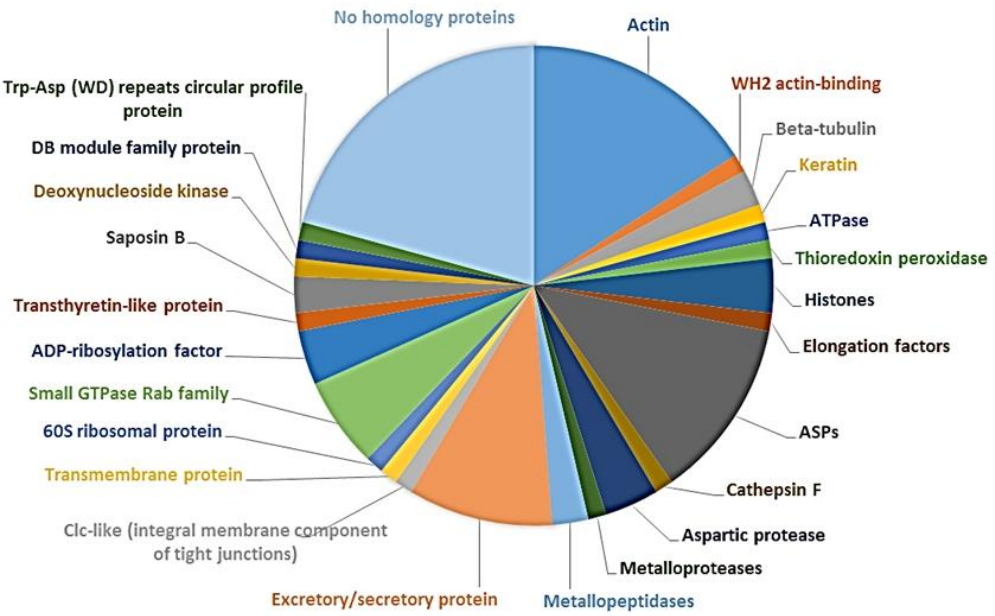


Figure 5.3. Pie chart summary of the protein groups identified in the exosome-enriched fraction after grouping the proteins according to their broad function.

5.3.3 Immunogenicity of the exosomes, exosome-free and total ES products

Exosomes, exosome-free and total ES products of *T. circumcincta* L4 were bound by IgG (Figure 5. 4) and IgA (Figure 5. 5) in sera from previously infected, but not in sera from uninfected sheep. There were protein bands, which were strongly recognized by IgG and IgA either only in the exosomes and the total ES products (e.g. area between 62 and 98 kDa), or only in the exosome-free and total ES products (e.g. area between 98 and 188 kDa or 28kDa area). The immunoblots suggest that the higher molecular weight molecules are bound more strongly by IgA, whilst a wider range of molecular weight molecules are bound by IgG.

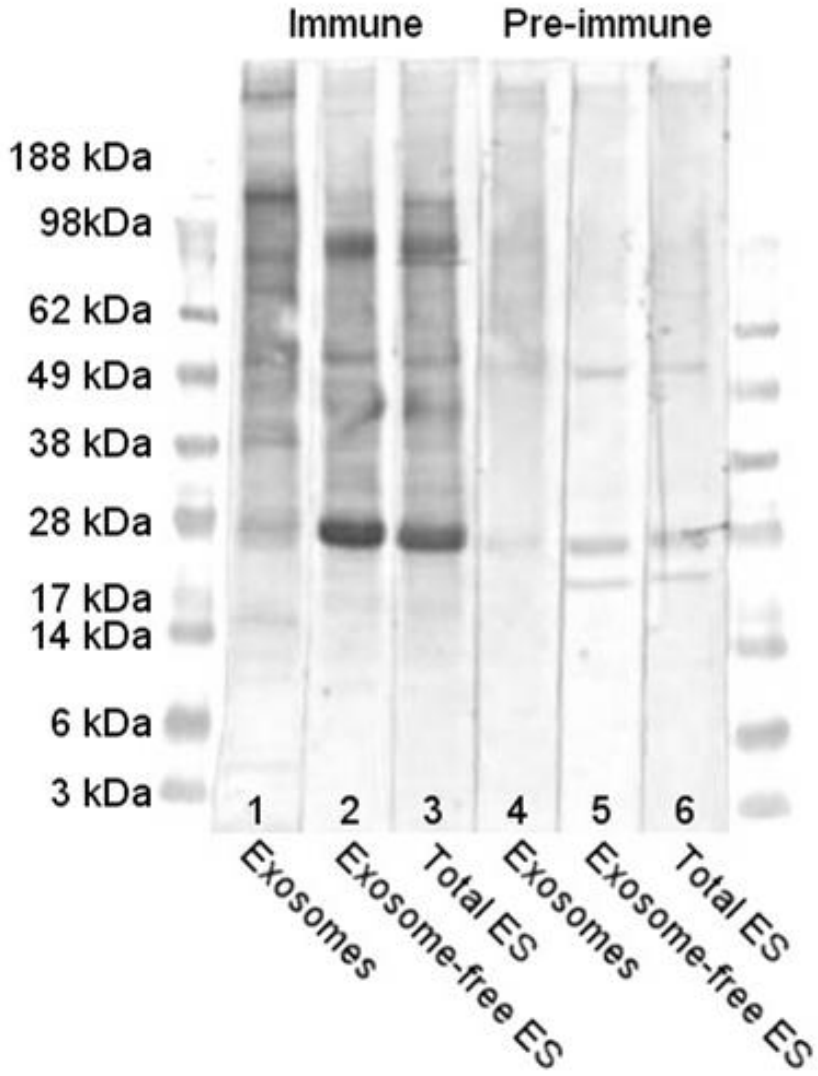


Figure 5.4. Immunoblot of exosomes, exosome-free ES and total ES products of *T. circumcincta* L4 stage against blood sera obtained from *T. circumcincta* trickle repeatedly infected sheep (lanes 1-3) and blood sera taken prior to the trickle infections (negative control, lanes 4-6). 2µg of each sample (10 µl total volume) were subjected to electrophoresis under reducing conditions followed by Western blot. IgG binding was examined after incubating in donkey anti-sheep/goat IgG horseradish peroxidase (HRP) conjugate (AbD Serotec). Peroxidase activity was revealed using DAB as substrate. There were proteins, which were strongly recognized by IgG either only in the exosomes and the total ES products or only in the exosome-free and total ES products.

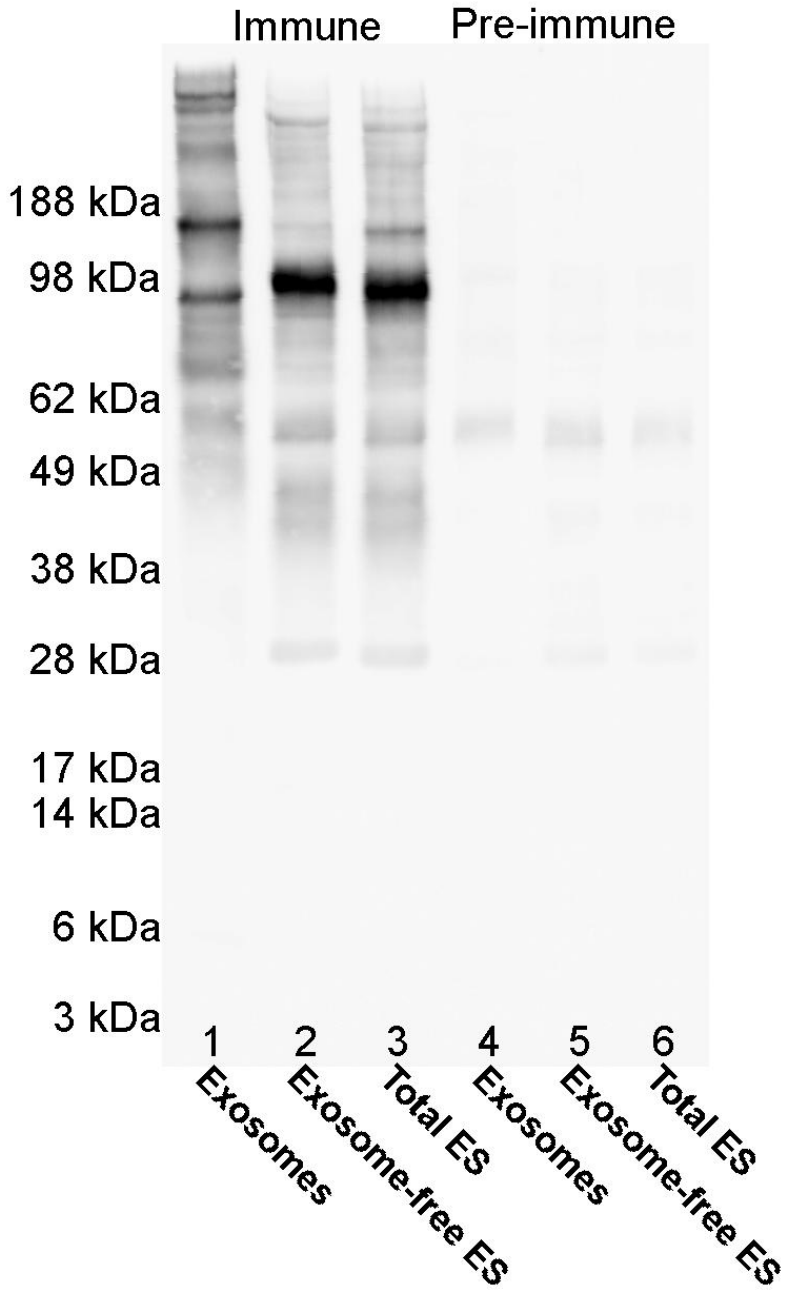


Figure 5.5. . Immunoblot of exosomes, exosome-free ES and total ES products of *T. circumcincta* L4 stage against blood sera obtained from *T. circumcincta* trickle repeatedly infected sheep (lanes 1-3) and blood sera taken prior to the trickle infections (negative control, lanes 4-6). 2µg of each sample (10 µl total volume) were subjected to electrophoresis under reducing conditions followed by Western blot. IgA binding was examined after incubating in mouse anti-bovine/ovine IgA (AbD Serotec). Peroxidase activity was revealed using ECL. There were proteins, which were strongly recognized by IgA either only in the exosomes and the total ES products or only in the exosome-free and total ES products.

5.4 Discussion

The work in this chapter demonstrated that exosomes are present in the ES products from *T. circumcincta* L4, which were harvested at 1-week post infection. This developmental stage was selected for the experiments, as it is most intimately associated with the abomasal glands of the host (Denham, 1969). Moreover, previous studies suggest that this stage is targeted by local humoral and cellular immune responses that are, in part, responsible for the development of protective immunity in sheep (Smith *et al.*, 1985, Smith *et al.*, 1986, Stear *et al.*, 1995, Strain *et al.*, 2002). The proteomics analysis showed that approximately 70% of the proteins detected in the exosomes samples were possibly unique to these components and that they were not identified in the exosome-free ES products samples. The remaining 30% of the proteins identified in the exosomes were identified in the exosome-free ES products. Finally, contents of the exosomes were found to be immunogenic, since they were bound strongly by IgG and IgA present in sera obtained from *T. circumcincta* infected sheep and the majority were not bound by immunoglobulin in sera from the animals prior to infection. For this reason, some of the proteins that were identified here could potentially be targeted in future vaccine and/or drug intervention studies aimed to control this important parasite.

After the successful purification of the EMVs from *T. circumcincta* L4 ES products, the TEM confirmed the presence of EMVs. The majority of the EMVs exhibited the morphological characteristics of typical exosomes (cup-shaped vesicles) with a size that varied from 50 to 100 nm (Thery *et al.*, 2006). Although exosomes have been reported in other parasitic helminths (Eger *et al.*, 2003, Marcilla *et al.*, 2012, Buck *et al.*, 2014), this is the first report, to our knowledge, of exosome production and release by a ruminant nematode. Following TEM confirmation of exosomes being present in the ES products, the protein profile of an exosome-enriched sample was compared with protein profiles of exosome-free samples and with total L4 ES products. The results suggested that the protein profile of the exosome-enriched sample was different to the profiles of the other two samples, and

that the exosome-enriched sample contained protein bands that were not identified in the exosome-free ES by gel electrophoresis and staining.

There are no studies in the literature that have performed proteomics analysis in both exosome-enriched and exosome-free ES products from helminths. The proteomic analysis revealed 82 proteins in the exosome-enriched sample. A total of 23 of these proteins were also found in the exosome-free ES products. Nevertheless, it is not known if this was observed due to insufficient exosome purification after the series of ultra-centrifugations. TEM in the exosome-free sample might have shown an insufficient purification, if exosomes were observed in this sample. This observation might also have been due to contamination of the samples during the loading of the samples to the NuPAGE® Novex® Bis-Tris 4–12% mini gel prior to the proteomics analysis. However, this is unlikely as a lane was left empty between the different samples (Figure 5. 2; Panel A). Another possible explanation is that maybe these proteins are released both individually and in the exosomes. The 82 proteins identified in the exosomes included, among others, structural proteins (e.g. actin, β-tubulin); metabolic proteins (e.g. peroxiredoxin); nuclear proteins (e.g. histone); activation-associated secreted proteins (ASPs); Rab GTPases; metallopeptidases; and several unknown proteins. The exosomes included several proteins that were not identified previously in *T. circumcincta* studies (Nisbet *et al.*, 2008, Smith *et al.*, 2009). The genes of 56% of the exosome proteins were identified in a previous transcriptomic study performed on *T. circumcincta* L4 harvested at 8 days post infection, but not in the L3 stage (Nisbet *et al.*, 2008). Only 16% of the exosome proteins detected here were identified after proteomics analysis of total ES products derived from *T. circumcincta* L4 harvested at 1-, 3- and 5 days post infection (Smith *et al.*, 2009).

In terms of comparison to exosomes of other organisms, the analysis showed that 76% of the exosome proteins identified in the current study were among molecules previously identified in the ExoCarta database (Mathivanan *et al.*, 2010, Marcilla *et al.*, 2012, Mathivanan *et al.*, 2012, Simpson *et al.*, 2012) and in other published studies relating to exosomes in helminth species (Marcilla *et al.*, 2012,

Bernal *et al.*, 2014). The remaining 24% included proteins such as, ASPs and ES proteins, which will be described in more detail below.

Actins and beta-tubulin are structural proteins which represented 16% and 2% of the proteins identified in the exosome-enriched sample, respectively, and they have been identified in exosomes of other organisms (Mathivanan and Simpson, 2009, Mathivanan *et al.*, 2012). Actins are associated with the cytoskeletal microfilaments and beta-tubulin with the cytoskeletal microtubules (Schappi *et al.*, 2014). Keratin, another cytoskeletal protein, has been also identified in exosomes originated from human saliva (Xiao and Wong, 2012) and represented 1% of the proteins identified. The identification of keratin in the proteomic analysis could indicate human contamination. However, the top hit after a tBLASTn search of the protein identified in the exosome-enriched sample against the whole nucleotide collection of NCBI was the *O. ostertagi* partial mRNA for keratin (AJ429146.1, 63% identities, E value= 2e-71). A subsequent tBLASTn search of the protein identified in the exosome-enriched sample against the human nucleotide collection resulted in no significant similarities. As a result, it is unlikely the identification of keratin in the exosome-enriched sample to be due to human contamination. WH2 actin-binding protein (1% of the proteins) has not been detected in the exosomes from other organisms in the past and has been associated with actin assembly (Carlier *et al.*, 2013). Nevertheless, there were other actin-binding proteins in the ExoCarta database, such as coronin and moesin. It has been suggested that these structural proteins might be associated with the exosome production (Wubbolts *et al.*, 2003).

The metabolic proteins that have been identified in the *T. circumcincta* exosome-enriched sample (Na/K-ATPase and thioredoxin peroxidase; 1% of the proteins each) have been found in the ExoCarta database. Ce-EAT-6 is a Na/K-ATPase and has been shown to interact with ATP and to have sodium:potassium-exchanging ATPase activity (Davis *et al.*, 1995). *C. elegans Ce-eat-6* mutations have been associated with paralysis of the pharyngeal muscles in *C. elegans* (Davis *et al.*, 1995). Moreover, studies in the trematode parasite, *Schistosoma mansoni*, have shown that the Na/K-ATPase has been associated with the acquisition of resistance to complement after the parasite has penetrated the skin (Tarrab-Hazdai *et al.*, 1997).

Nevertheless, there are no studies to show whether or not this ATPase is found in the exosomes of this species. This protein in *T. circumcincta* exosomes might be proposed to aid in the parasites' establishment in mucosa or gastric gland.

Thioredoxin peroxidase (peroxiredoxin) was identified in the *T. circumcincta* exosome-enriched sample. This is an antioxidant enzyme that is considered as a damage-associated molecular pattern molecule (DAMP), but also as a pathogen-associated molecular pattern molecule (PAMP) (Medzhitov, 2007, Robinson *et al.*, 2010b). Peroxiredoxins are thought to aid in the survival of parasites against reactive oxygen species generated by the immune system of the host (Gretes *et al.*, 2012). Moreover, it has been proposed that helminth peroxiredoxins are homologous to host DAMPs and are responsible for the modulation of the immune response (Robinson *et al.*, 2010b, Dalton *et al.*, 2013). In *F. hepatica*, these molecules have been shown to help direct the immune system towards a Th2-type response that is thought to be favourable for the parasites' development in the host (Robinson *et al.*, 2010a, Dalton *et al.*, 2013). Peroxiredoxin has also been found in the exosomes of *F. hepatica* and in *E. caproni* (Marcilla *et al.*, 2012). Peroxiredoxin has been proposed as a potential vaccine candidate in *Fasciola* spp., due to its immunomodulatory properties (Robinson *et al.*, 2010a, Robinson *et al.*, 2010b). Nevetheless, vaccine trials with recombinant peroxiredoxin in goats and buffaloes did not show significant levels of protection (Mendes *et al.*, 2010, Raina *et al.*, 2011).

A notable proportion (12%) of the proteins identified in the exosome-enriched fraction were found to belong to the ASP group. These proteins have not been found in the exosomes of other organisms in the ExoCarta database, including the databases relating to the helminths *F. hepatica*, *E. caproni* and *Dicrocoelium dendriticum* (Marcilla *et al.*, 2012, Bernal *et al.*, 2014). This could be explained by the fact that ASPs are a nematode-specific group of proteins (Parkinson *et al.*, 2004). Nevertheless, SCP/TAPS were also described in the trematode parasite *Schistosoma mansoni* (Chalmers *et al.*, 2008). This venom allergen-like (SmVAL) protein family in *S. mansoni* consists of 29 members, a proportion of which can be found in the ES products and the remaining within the parasite (Yoshino *et al.*, 2014). As mentioned in previous chapters, the precise function of nematode ASPs is still unknown.

However, it is suggested that these proteins might act as virulence factors that manipulate host immune responses and contribute to parasite survival in the host (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999, Asojo *et al.*, 2005). Moreover, ASPs have been identified as vaccine candidates against several parasitic nematodes, including *T. circumcincta* (Ghosh *et al.*, 1996, Kooyman *et al.*, 2000, Geldhof *et al.*, 2002, Nisbet *et al.*, 2013). Here, 10 ASPs were identified in the exosome-enriched sample (*tdc00434*, *tdc00460*, *tdc00472*, *tdc00533*, *tdc00656*, *tdc00942 tdc02274*, *tdc02406*, *tdc02610* and *tdc02887*). It has been suggested that the proteins secreted via the exosomes might or might not have a signal peptide in their sequence (Liegeois *et al.*, 2006, Marcilla *et al.*, 2012). Based on that, the aforementioned ASPs were searched for the presence of a signal peptide in their sequences. The former 8 ASP molecules had a signal peptide in their sequence (*tdc00434*, *tdc00460*, *tdc00472*, *tdc00533*, *tdc00656*, *tdc00942 tdc02274*, *tdc02406*), but not the latter 2 (*tdc02610* and *tdc02887*). Moreover, the genes that encode 2 of the ASPs were amplified in Chapter 3, in particular *tdc00533* and *tdc002274*. The former gene was expressed in adult worms only whilst the latter in all the stages apart from the egg and L4 stage of the parasite (Chapter 3, Figure 3.5). In addition, those proteins were not the ASPs identified in a previous study that tried to identify potential vaccine targets in ES products of L4 *T. circumcincta* harvested at 5 days post infection (Nisbet *et al.*, 2010b). This might have been due to the low concentration of exosomes in the total ES products. As a result, the novel ASPs revealed from this proteomic analysis might be potential targets for the future control of *T. circumcincta*.

Another large group of proteins, that represented 10% of the proteins identified in the exosome-enriched material and have not been previously identified in the exosomes of other organisms, are ES proteins that have been ascribed no specific function. Those identified did not have any motifs in their sequences. However, TDP01869 was found to be homologus of the *H. contortus* 15 kDa ES protein (Schallig *et al.*, 1997b); HCP02856, HCP06214 and TDP00176 were homologues of the *T. circumcincta* 20 kDa ES protein (Nisbet *et al.*, 2013); TDP00436 and TDP00589 were homologues of the *O. ostertagi* ‘putative L3 ES protein (De Maere

et al., 2002); and OOP00884 and TDP00713 were homologues of the *T. colubriformis* 30 kDa glycoprotein (Savin *et al.*, 1990). TDP01869, HCP06214 and TDP00589 had a signal peptide in their sequences, whilst HCP02856, TDP00176, TDP00436, OOP00884 and TDP00713 did not have a signal peptide in their sequences. The homologue proteins *H. contortus* 15 kDa ES protein, *T. circumcincta* 20 kDa ES protein, *O. ostertagi* 'putative L3 ES protein and *T. colubriformis* 30 kDa glycoprotein have been described as potential vaccine candidates in previous studies. In particular, in an immunisation trial in sheep using the two native *H. contortus* ES proteins (the 15 kDa and 24 kDa ES proteins), significant protection against challenge was observed in vaccinated sheep when compared with challenge control animals (Schallig *et al.*, 1997a). Furthermore, the *T. circumcincta* 20 kDa ES protein is one of the components of an eight-protein recombinant protein 'cocktail' that has been shown to induce significant levels of protection in vaccinated lambs compared to challenge control animals (Nisbet *et al.*, 2013). The *O. ostertagi* 'putative L3 ES protein' has been proposed as potentially protective antigen because it was recognized by antibodies from "immune" calves (De Maere *et al.*, 2002) but it remains to be tested in an immunisation trial. The *T. colubriformis* 30 kDa glycoprotein was found to be protective in guinea pigs against challenge and was found to reduce worm burdens by 59% in vaccinates compared to challenge control animals (Savin *et al.*, 1990). The function of the above proteins is unknown, however their involvement in several successful vaccine trials indicates their potential as valid vaccine candidates.

Cathepsin F (Tci-CF-1) was found here in the exosomes of *T. circumcincta* (1%). Tci-CF-1 is the most abundant molecule to be identified in the ES products of *T. circumcincta* larvae (including those harvested at 5, 6 and 9 days post infection) and has been found to be a target of IgA responses in previously infected sheep (Redmond *et al.*, 2006). A close (40% homology) homologue of this protein have been identified in the exosomes of *F. hepatica* (Cathepsin L - FheCL; Marcilla *et al.*, 2012). FheCL has been associated with tissue penetration during the larvae migration through the host tissue (Dalton and Heffernan, 1989), nutrition (Dalton and Heffernan, 1989, Smith *et al.*, 1993b) and/or immunomodulation (Chapman and

Mitchell, 1982, Dalton and Heffernan, 1989, Smith *et al.*, 1993a). A more recent study has shown that FheCL suppressed Th1 immune responses by suppressing the production of IFN-γ (O'Neill *et al.*, 2001). It has been suggested that Tci-CF-1 has similar immunomodulatory function (Redmond *et al.*, 2006). Tci-CF-1 and FheCL have been tested in vaccine trials, which showed significant levels of protection against *T. circumcincta* in lambs (Nisbet *et al.*, 2013) and *F. hepatica* in calves (Dalton *et al.*, 1996), respectively.

Rab GTPases represented 6% of the proteins found in the exosomes analysed here. These proteins have been highlighted in previous exosome studies in helminths (Marcilla *et al.*, 2012) and were identified in other organisms in the ExoCarta database search. In 2010, there were 40 Rab proteins in the ExoCarta database from various studies on exosomes (Mathivanan *et al.*, 2010). Similar numbers are present in the database currently. Rab proteins are the largest family of small GTPases and have been associated with the exosome secretion pathway in cells, for example Rab27 in Hela cells (Ostrowski *et al.*, 2010), Rab35 in oligodendroglial cells (Hsu *et al.*, 2010) and Rab11 in erythroleukemia cell lines (Savina *et al.*, 2005). Studies have shown that different Rab proteins are involved with different stages of the exosome secretion pathway (Stenmark, 2009). For example, RAB4, RAB5 and RAB11 are involved in the early stages, whilst RAB7 and RAB9 in the late stages of the exosome secretion pathway (Stenmark, 2009).

Finally, the group of unknown proteins with no homology hits identified after the BLAST search comprised the largest proportion of proteins in the exosome-enriched fraction (21%). These proteins did not have any motifs recognized in their sequence that would indicate a potential function. A previous study has also identified proteins with no homology and unknown function (17% of the exosome proteins) in *D. dendriticum*- derived exosomes (Bernal *et al.*, 2014).

An important finding here was that components of the exosome-enriched samples are immunogenic after incubating the samples with serum from experimentally trickle-infected sheep over a period of 6 weeks. The immunoblots that were performed revealed that the exosome proteins were bound by IgG and IgA.

This is an indication that the exosomes are actually secreted *in vivo* and are not an artefact from the *in vitro* culture of the parasites. There were not any studies in the literature that have tested the immunogenicity of the exosomes originated from other parasites. The results of the immunoblots suggest that the higher molecular weight molecules are bound more strongly by IgA, whilst a wider range of molecular weight molecules are bound by the IgG. Thus, it can be suggested that some of the molecules found in the exosome-enriched samples could be potential vaccine targets since they are recognised by the immune system of the host undergoing a protective response.

In conclusion, exosomes are released by *T. circumcincta* 1-week post infection. The proteomic analysis revealed several proteins with potential immunomodulatory function, supporting the rationale proposed in the past that helminths potentially use exosomes to simultaneously deliver multiple antigens into host cells and regulate their function (Dalton *et al.*, 2013). A study for the future would be to determine whether *T. circumcincta* exosomes contain miRNAS and, if so, whether they are used to regulate host gene expression similar to other nematodes (Buck *et al.*, 2014). Finally, the binding of proteins within the exosome-enriched fractions by IgA and IgG of previously infected sheep confirmed their immunogenicity. Future experiments could focus on the purification and characterisation of exosomes from other time points after *T. circumcincta* infection (e.g. 1-, 3- and/or 5 dpi), since previous studies have shown that early host responses affect establishment of the parasite (Stear *et al.*, 1995, Stear *et al.*, 1999a, Stear *et al.*, 1999b). In addition, the protein profile of *T. circumcincta*'s ES products is different among the above time points (Balic *et al.*, 2003, Smith *et al.*, 2009), which suggests that the proteins included in the exosomes might also be different.

Chapter 6: General discussion

The major aim of this PhD project was the development of an RNAi platform in the ovine parasitic nematode *T. circumcincta*; the ambition being to use this as a method to screen potential vaccine candidates against this parasite. The majority of the target-genes for the RNAi belonged to the Activation-associated Secreted Proteins (ASPs) group, which are vaccine candidates in several parasitic nematodes. These proteins have been tested, with considerable success, in vaccine trials against several nematode species, including *Haemonchus contortus* (Schallig *et al.*, 1997a, Schallig and Van Leeuwen, 1997, Kooyman *et al.*, 2000), *Ancylostoma caninum* (Ghosh *et al.*, 1996, Sen *et al.*, 2000, Goud *et al.*, 2004) and *Ostertagia ostertagi* (Geldhof *et al.*, 2002, Geldhof *et al.*, 2004). Recently, Nisbet *et al.* (2013) conducted a vaccine trial in which they immunised sheep with a vaccine-cocktail comprising eight recombinant proteins (six expressed in bacterial cells and two in yeast), one of which was an ASP, Tci-ASP-1. The latter had been identified as being the target of local mucosal IgA responses in infected lambs (Nisbet *et al.*, 2010b). The results of the vaccine trial were promising in that significant levels of protection were induced in immunised lambs compared to control animals (Nisbet *et al.*, 2013). However, Tci-ASP-1 was only one component of the vaccine and testing each one or selecting more defined 'cocktails' would be labour-intensive and expensive. The use of RNAi could provide a more rapid and specific method to help determine molecules that are critical to the survival of *T. circumcincta* inside the host. For example, components of the current eight-protein 'cocktail' could be targeted by RNAi to seek detrimental or lethal phenotypes, initially *in vitro*, but, if needed, *in vivo*. It is possible that the impact of RNAi conducted in the L3 stage would not manifest itself until the later parasitic stages. In this context, Samarasinghe *et al.*, (2011) established that RNAi-mediated knockdown of a gene encoding an intestinal aminopeptidase (H11) manifested, phenotypically, as a reduction in egg output and worm numbers at the end of the infection period but had no obvious effect on L3 viability and infectivity *in vitro*. Exploitation of RNAi technology could facilitate understanding of the biological function of ASPs, which is a major knowledge gap at the current time.

Although development of RNAi in *T. circumcincta* may on the surface appear straightforward, at the outset, there were several issues to be considered and resolved in this parasitic nematode. RNAi was initially described in the free-living nematode *C. elegans* (Fire *et al.*, 1998). In this model, RNAi was used broadly and successfully for the functional analysis of its genome (Maeda *et al.*, 2001, Kamath *et al.*, 2003). Following the success in *C. elegans*, the efficacy of RNAi was examined in a number of parasitic nematode species, including *Nippostrongylus brasiliensis* (Hussein *et al.*, 2002), *Trichostrongylus colubriformis* (Issa *et al.*, 2005), *H. contortus* (Geldhof *et al.*, 2006b), and *O. ostertagi* (Visser *et al.*, 2006). Unfortunately, the application of RNAi in parasitic nematodes was not as successful as in *C. elegans* (Britton and Murray, 2006, Geldhof *et al.*, 2007). The difficulty lay in the variability of the results and, in particular, in the inconsistency of the silencing effect among different parasite species, different target-genes, and even among biological replicates targeting the same gene (Geldhof *et al.*, 2006b, Visser *et al.*, 2006). The reason for these discrepancies was not clear. Several suggestions had been proposed for the inconsistency and, included, among others, inappropriate dsRNA delivery method (Viney and Thompson, 2008), absence of some of the essential genes required for successful RNAi (Viney and Thompson, 2008) and/or the expression site of the target gene (Samarasinghe *et al.*, 2011). Where RNAi had failed, it was not known whether the dsRNA had been taken up by the parasites or if the RNAi pathway was activated in response to exposure to the gene-specific dsRNA or, whether the pathway was at a constant level of activation.

At the time of commencing this study, there were no reports in the literature regarding transcript levels of the RNAi pathway genes and their response, if any, after the introduction of exogenous gene-specific dsRNA in parasitic nematodes. If the transcription of some of the RNAi pathway genes was stimulated by dsRNA exposure, measurement of these could shed light on the consistency of the method as these RNAi pathway genes could be used as internal controls that would indicate activation of the pathway in response to dsRNA exposure. In the first part of the project (Chapter 2), attempts were made to develop these internal controls that could be applied to help understand inconsistencies in the subsequent application of RNAi

in *T. circumcincta*. To accomplish this, *C. elegans* was used as a model organism because of the consistency of results reported in the past (Maeda *et al.*, 2001, Kamath *et al.*, 2003, Geldhof *et al.*, 2006a). Kamath *et al.*, 2003 applied RNAi and successfully inhibited approximately 86% of the 19,427 predicted genes of *C. elegans*. Here, two genes, *Ce-cpr-4* and *Ce-sod-4*, were targeted to expose worms to exogenous dsRNA and to examine how three RNAi pathway genes responded to exposure. *Ce-cpr-4* is a cysteine protease-encoding gene shown to be consistently susceptible to RNAi (Geldhof *et al.*, 2006a), whilst *Ce-sod-4* is a superoxide dismutase-encoding gene which has been consistently refractory to RNAi (David Knox, personal communication). The next step was to choose candidate control genes to study from the RNAi pathway. These were selected based on their function in the pathway and on how conserved they were among nematodes in order to be able to be used in *T. circumcincta* in later experiments. As a result, three RNAi pathway genes were chosen; *Ce-dcr-1, Ce-ego-1* and *Ce-rsd-3*. These pathway genes were conserved among the parasitic nematodes (Dalzell *et al.*, 2011) and represented different parts of the RNAi pathway. After a series of optimisation experiments, RNAi was found to be successful after 1 h of soaking in gene-specific dsRNA for *Ce-cpr-4*, but not for *Ce-sod-4* (Chapter 2). The rationale for the duration of the soaking period was based on the fact that RNAi is considered an ancient cellular anti-viral mechanism (Vance and Vaucheret, 2001) and hence should be activated shortly after exposure to the gene-specific dsRNA. The transcript levels of the candidate pathway genes were estimated in worms with activated and inactivated RNAi pathways (*Ce-cpr-4* and *Ce-sod-4*, respectively) and in untreated worms (soaked in 1xPBS) with end-point RT-PCR and with qRT-PCR (Chapter 2). The results showed that the pathway genes could not be used as controls that would indicate activation or not of the RNAi pathway as the transcript levels were stable regardless of exposure to exogenous dsRNA and whether or not this induced specific transcript knockdown. These results have been peer reviewed and published (Tzelos *et al.*, 2013). A potential reason for this apparent unresponsiveness might be the presence of different small RNA pathways that are known to be used for the regulation of gene expression in *C. elegans* (Fischer, 2010). In particular, apart from the RNAi pathway that uses siRNAs produced by exogenous dsRNA (Fire *et al.*,

1998), there had also been revealed endogenous siRNAs that originate from endogenous loci and potentially regulate transcript levels of genes (Duchaine *et al.*, 2006, Lee *et al.*, 2006, Guang *et al.*, 2008). Furthermore, there are the microRNAs (miRNAs) which were found to be involved in the inhibition of translation and/or the stability of target mRNAs (Lee *et al.*, 1993, Pasquinelli *et al.*, 2000, Reinhart *et al.*, 2000). The presence of different small RNA pathways that regulate gene expression by using the same machinery might explain the stable transcript levels of the RNAi pathway genes selected here. Future studies should examine whether other genes of the RNAi pathway [for example, *Ce-eri-1* (Enhanced RNA interference)] could be used as internal controls. The protein encoded by this gene exhibits siRNase activity and negatively regulates RNAi (Kennedy *et al.*, 2004). Additional genes such as this were not included in the current study because, at the time, this gene could not be found in the partial genome sequence of *T. circumcincta* that was available. This gene has been found in subsequent bioinformatics searches of updated *T. circumcincta* genome datasets and could be examined in future. Another useful experiment would be to examine whether or not the protein levels of the three candidate pathway genes could be used as markers for the activation of the RNAi pathway. This could be achieved by raising antibodies against the proteins that are encoded by the genes that were tested in this project (i.e. Ce-DCR-1, Ce-EGO-1 and Ce-RSD-3) and examine their levels when RNAi is successful or not.

Next, efficacy of the RNAi method in *T. circumcincta* was examined (Chapter 4). The first step here was to select target genes for the RNAi. ASP genes were chosen for this purpose because, as mentioned before, they are valid vaccine candidates and their precise function is yet to be revealed. The use of RNAi in these proteins not only could reveal their biological function, but also determine whether Tci-ASP-1 is a protective component of the recombinant vaccine that was tested recently (Nisbet *et al.*, 2013). Several ASP molecules had been identified previously in transcriptome and the ES products of *T. circumcincta* L3 and L4 stages (Nisbet *et al.*, 2008, Nisbet *et al.*, 2010b). At the start of this study, no work had been published that sought to identify and characterise the diversity of ASPs in *T. circumcincta*. Such an analysis would shed light on the potential role of these molecules in the

parasite, reveal novel vaccine candidates and, importantly, would be of great benefit in selecting genes that would be targeted in the subsequent RNAi experiments. A bioinformatics search was conducted using the database of Nembase4 (http://www.nematodes.org/nembase4/) to find molecules with the ASP motif in their sequence (InterProScan: IPR014044). The results showed that *T. circumcincta* has the second highest numbers of ASP-encoding genes, described to date, in the parasitic nematodes after *A. caninum*. It was found that *T. circumcincta* had 131 ESTs that formed 58 ASP-encoding genes. To examine which of these might be excreted or secreted by the parasite, these 58 sequences were searched for a signal peptide sequence. Because the sequences in the Nembase4 database do not represent the full-length of the genes, some signal peptide sequences may have been missed, however, 21 proteins were identified. These represented all the different types of ASPs: double-domain (DD), N-type single-domain (N-type SD) and C-type- single-domain (C-type SD). Unique areas were sought in each sequence to design primers specific for each gene, which later would be employed in a stage-specific end-point PCR. Unique areas were identified only for 19 genes and these were studied further. The transcripts of only 12 out of 19 genes were amplifiable in a stage-specific end-point PCR. The ASP transcripts that could be detected by PCR exhibited different developmental stage transcription patterns, with some amplified only from stages found inside the host (xL3, L4, adults), whilst others were amplifiable in cDNA derived from pre-parasitic as well as parasitic stages of *T. circumcincta*. The expression patterns of the 12 genes that were amplified were similar but not identical with the patterns of their homologues in other species, particularly *O. ostertagi*, *H. contortus* and *A. caninum* (Chapter 3). Of note, the transcript of seven of those ASP genes was detectable in CO_2-exsheathed L3, but not larvae exsheathed using a method that involves NaClO, suggesting that the L3 exsheathment protocol can have an impact on gene expression. However, it is not known whether the exsheathment method has an effect on genes other than ASPs. Based on the findings here, it is essential that the effect of the exsheathment protocol applied is routinely tested in future studies of transcript abundance.

The subsequent selection of RNAi target-genes in *T. circumcincta* (Chapter 4) was based, in part, on the results above. The target genes included five ASP genes (*tdc00462*, *Tci-asp-1*, *tdc00691*, *tdc00879* and *tdc01479*), *Tci-mif-1* (macrophage migration inhibitory factor-like) and *Tci-saa-1* (surface associated antigen). Three of the ASP genes studied here (*tdc00462*, *Tci-asp-1* and *tdc00879*) had been found to be transcribed in the parasitic stages xL3, L4 and adults, suggesting a potential role in transition to parasitism, immune evasion and establishment as has been proposed in the past (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999). They represented all the types of ASP, namely C-type SD, N-type SD and DD, respectively. The remaining two ASPs (*tdc00691* and *tdc01479*) were transcribed in all stages of the parasite, which suggests another function potentially irrelevant with parasitism or maybe they are not translated or released until parasitism. These proteins were DD and N-type SD ASPs, respectively. The additional target-genes (*Tci-mif-1* and *Tci-saa-1*) were selected so that the study encompassed target genes from other protein families and because these proteins were components of the recombinant vaccine-cocktail that was recently tested in *T. circumcincta* (Nisbet *et al.*, 2013). These are transcribed in the L3 stage, and thus, they were valid targets for this study (Zhan *et al.*, 2004, Nisbet *et al.*, 2009, Nisbet *et al.*, 2010a). Previous studies showed that *Tci-mif-1* is a potentially immunosuppressive molecule (Nisbet *et al.*, 2010a) and *Tci-saa-1* is an immunogenic homologue protein of a protective antigen from *A. caninum* (Zhan *et al.*, 2004, Nisbet *et al.*, 2009).

The RNAi protocol optimised in *C. elegans* was then applied to *T. circumcincta*. The RNAi experiments showed a successful silencing of four out of the seven genes originally selected, which illustrates the inconsistencies that had been reported in other species (Britton and Murray, 2006, Geldhof *et al.*, 2007). In this study, the L3 were soaked in dsRNA for 1 hour. The genes for which RNAi was unsuccessful might have been successfully silenced after longer soaking periods. However, this hypothesis was not tested here. After the experiments mentioned above, *Tci-asp-1* was studied in more detail because it is a component of the *T. circumcincta* recombinant vaccine (Nisbet *et al.*, 2013). Nisbet *et al.* (2010b) did not manage to detect the protein or transcript of *Tci-asp-1* in the NaClO exsheathed L3

previously. Nevertheless, since the *asp-1* transcript was detected here in CO_2 exsheathed L3, attempts were made to detect the protein by Western blot in L3 exsheathed in this manner. The ASP-1 protein could not be identified in the CO_2 exsheathed L3 and as a result, the effect of RNAi on Tci-ASP-1 protein level could not be examined. The results of the western blot showed that the protein could not be detected 24, 48, 72 nor 96 h after CO_2 exsheathment. Potential reasons for this might be that a trigger of the host might be required to initiate the translation of *Tci-asp-1* or that the gene is transcribed but not translated in this stage of the parasite. It would be of interest to soak infective L3 in *Tci-asp-1* encoding dsRNA prior to using these to infect lambs and comparing the outcome of infection with control larvae that are not exposed to the dsRNA. This approach may reveal a phenotypic difference in the outcome of infection and provide an indication of the effect, if any, of the Tci-ASP-1 protein product on the immune response to the parasite. Evidence for the latter could be sought using comparative immunohistochemistry (to identify immune cells recruited within the mucosa) combined with immunological (cellular and humoral) and transcriptomic approaches aimed at identifying any specific changes in the mucosal responses in terms of gene expression.

It has been shown previously that RNAi has a temporary effect which means that, in the absence of dsRNA, the transcript can recover to normal levels after a period of time (Domeier *et al.*, 2000). The duration of the silencing effect on *Tci-asp-1* was estimated to be 24 h after the removal of the gene-specific dsRNA. This is short period of time compared to that observed previously in trematode parasites (specifically, *Schistosoma mansoni*) in which the silencing effect was observed to last for at least up to 28 days (Boyle *et al.*, 2003). However, as in other studies in parasitic nematodes, the RNAi effect proved to be inconsistent here and the silencing effect of *Tci-asp-1* specific dsRNA was not completely reproducible in different experiments (Chapter 4). Two potential reasons for the inconsistencies are; the absence or non-functional RNAi pathway genes and/or inappropriate dsRNA delivery method (Knox *et al.*, 2007, Viney and Thompson, 2008). The former was addressed with a bioinformatics search to identify RNAi pathway genes in the *T. circumcincta* genome, and the latter in an experiment in which fluorescently labelled

dsRNA and siRNA were used to confirm uptake. The bioinformatics search showed that there were 50 out of 74 RNAi pathway genes, which are considered to be essential for successful RNAi in *C. elegans* (Dalzell *et al.*, 2011), present in the existing databases of the *T. circumcincta* genome. At the time of the search, the parasite's genome was not fully annotated which could explain the lack of 24 genes from the *T. circumcincta* genome and led to a second bioinformatics search of the *H. contortus* genome (Laing *et al.*, 2013), this having been updated since the search by Dalzell *et al.*, 2011. This search revealed 19 genes that were not identified previously (Dalzell *et al.*, 2011), including *rde-4*. Previous searches of the *H. contortus* genome and EST datasets had failed to identify a homologue of *rde-4* (Geldhof *et al.*, 2006b, Dalzell *et al.*, 2011). RNAi is inoperative in *C. elegans* mutants lacking *rde-4* (Tabara *et al.*, 1999) and these data led Knox *et al*, (2007) to suggest that RNAi in parasitic helminths may be RDE-4 independent and lack of this gene might be a reason for the inconsistencies. An *rde-4* homologue was not identified in *T. circumcincta* but, given the finding in *H. contortus*, probably reflects the extent of gene coverage in the ongoing genome annotation and a homologue is likely to be found. The experiments with the fluorescently-labelled dsRNA and siRNA showed that both dsRNA and siRNA were taken up by the parasites after soaking the L3 for more than 48 h with the intensity of the fluorescence with the siRNAs being greater compared to that following soaking in dsRNA. However, it cannot be concluded that the siRNA delivery was better than the dsRNA. Although the volume and the concentration of the FITC-labelled siRNAs and dsRNA solutions was the same, the molarity of the solutions was different. The population of the individually FITC-labelled dsRNAs, which were 218bp long, was approximately 10 times less than the individually FITC-labelled siRNAs. This might have been the reason for the differences in the intensity of the fluorescence. The uptake after 1 and 24 h of soaking could not be estimated due to auto-fluorescence displayed by the untreated L3. Similar results were observed in *H. contortus* in which the uptake of dsRNA was obvious after 72 and 96 h (Geldhof *et al.*, 2006b).

Since the silencing effect of *Tci-asp-1* specific dsRNA was not reproducible in different biological repeats, an alternative protocol was tried in which larvae were

soaked in hsiRNA (Landmann *et al.*, 2012). Soaking exsheathed L3 in hsiRNAs was previously used successfully in the filarial nematode, *B. malayi* (Landmann *et al.*, 2012). The results of this study showed successful and consistent RNAi in three target genes of *B. malayi* (Landmann *et al.*, 2012), however it was unsuccessful in *T. circumcincta*. A possible breakthrough finding here was the observation that the storage period of the larvae, prior to use in the RNAi experiments, affected the susceptibility of the parasites to the procedure. When 'fresh' (stored for a week) and 'old' (stored for a year) larvae, both populations being viable as judged by motility and structure, were soaked in gene-specific dsRNA, successful silencing was observed only with the 'fresh' parasites (Chapter 4). This experiment was reproduced in quadruplicate and the results were consistent in all. A time-course experiment was then conducted to test for how long the larvae were susceptible after the culture. It was found that the larvae became refractory to RNAi after being stored at 4°C for a month. These results could not be compared with other parasitic nematodes in which RNAi was applied. The storage period of the worms, prior to their use, was not mentioned in previous RNAi studies in parasitic nematodes probably because it was not considered as a potential factor that would affect RNAi susceptibility (Geldhof *et al.*, 2006b, Visser *et al.*, 2006, Samarasinghe *et al.*, 2011). When the protocol with the hsiRNA was applied on 'fresh' larvae, knockdown was successful but the silencing effect was not as robust as with dsRNA. Similar results were observed in the flour beetle, *Tribolium castaneum*, when the efficacy of dsRNA and siRNA was compared (Wang *et al.*, 2013). The results of this study have shown that siRNAs were less effective than their corresponding dsRNA, potentially due to faster degradation of the siRNAs (Wang *et al.*, 2013). Nevertheless, there are no similar studies conducted in helminths or other endoparasites. Based on the results of this study, it can be suggested that the inconsistencies observed previously could be due to variable storage periods of larvae prior to use in RNAi. Storage protocols therefore merit further study in future RNAi studies in parasitic nematodes. The storage period of the parasites prior to their use and previous observations that susceptibility to RNAi depends on the expression site of the gene (Samarasinghe *et al.*, 2011) might lead to more optimal protocols in the future. The majority of the results from Chapter

4 were peer reviewed and published together with the results from Chapter 2 (Tzelos *et al.*, 2013).

The last part of this PhD study focused on exosomes released in the ES products of *T. circumcincta* (Chapter 5). Exosomes are a specific cell product and contain proteins that generally belong to a few specific families, such as actins and Rab GTPases, which are common in exosomes from different cell types (Mathivanan *et al.*, 2010, Mathivanan *et al.*, 2012). Moreover, exosomes were found to contain mRNA and miRNA and some of these mRNAs can be translated in target cells (Valadi *et al.*, 2007). This is a field of research that is relatively new and, already, exosomes have been identified in parasitic helminths (Marcilla *et al.*, 2012, Montaner *et al.*, 2014) and protozoa (Silverman *et al.*, 2010); however there were no published data regarding characterisation of exosomes in *T. circumcincta* and parasitic nematodes of veterinary importance in general. Given that exosomes are secreted by trematode parasites (Marcilla *et al.*, 2012), have the capability to modulate innate and adaptive immune responses of the host (Silverman *et al.*, 2010, Buck *et al.*, 2014) and play an important role in cell-cell communication, it was relevant to examine the possibility that *T. circumcincta* releases exosomes and if so, define their content.

After confirmation by TEM that exosomes were present in the ES products, a proteomics analysis (SOSPA) showed that 82 different proteins could be detected in exosomes and 132 proteins in the exosome-free ES products. A total of 23 of these proteins were common in both samples. There have been three different exosome purification protocols described, to date. The majority of the studies followed the differential centrifugation protocol (Thery *et al.*, 2006, Marcilla *et al.*, 2012, Buck *et al.*, 2014), and the same protocol was followed in this PhD project. The remaining two protocols are not used broadly and include: rate zonal centrifugation and immunocapture (Mathivanan *et al.*, 2010). The former protocol uses the flotation density in a sucrose gradient to isolate exosomes, whilst the latter is uses antibodies to isolate purified exosomes (Mathivanan *et al.*, 2010). There were no studies in the literature that have performed proteomics analysis in both exosomes and exosome-free ES products to examine whether there were any common proteins in the

exosomes and the exosome-free ES products. There is a possibility that the purification of the exosomes was insufficient and that there was some contamination of exosomes in the exosome-free ES products, or *vice versa*. This could have been addressed by performing TEM in the exosome-free ES products to validate the presence or absence of exosomes. In summary, 76% of the exosome proteins were among molecules previously identified in the ExoCarta database (Mathivanan *et al.*, 2010, Marcilla *et al.*, 2012, Mathivanan *et al.*, 2012, Simpson *et al.*, 2012) and in exosomes related studies in helminths (Marcilla *et al.*, 2012, Bernal *et al.*, 2014). Here, the exosomes contained several proteins that have possible immunomodulatory functions and potentially are used to aid the establishment of the parasite in the host. Some examples include: the metabolic proteins Na/K-ATPase (Tarrab-Hazdai *et al.*, 1997) and thioredoxin peroxidase (Robinson *et al.*, 2010b, Dalton *et al.*, 2013); ASPs (Hawdon *et al.*, 1996, Hawdon *et al.*, 1999, Asojo *et al.*, 2005); Cathepsin F (Chapman and Mitchell, 1982, Dalton and Heffernan, 1989, Smith *et al.*, 1993a); and, ES proteins that have not been ascribed a specific function (Savin *et al.*, 1990, Schallig *et al.*, 1997a, De Maere *et al.*, 2002, Nisbet *et al.*, 2013). The identified ASPs in the exosomes had not been found in the exosomes of other organisms in the ExoCarta database, including the helminths *F. hepatica*, *E. caproni* and *Dicrocoelium dendriticum* (Marcilla *et al.*, 2012, Bernal *et al.*, 2014). This can be explained by the fact that the ASPs are a nematode specific group of proteins (Parkinson *et al.*, 2004). Moreover, some of the ASPs identified in the exosomes were not identified in a previous study that tried to identify potential vaccine targets in the ES products of L4 *T. circumcincta* (Nisbet *et al.*, 2010b) and, hence, may themselves be possible vaccine candidates. The presence of the proteins described above in exosomes supports the suggestion that helminths potentially use these vesicles to simultaneously deliver multiple proteins into host cells to regulate their function (Dalton *et al.*, 2013). Thus far, there is no evidence in the literature of heterogeneity in exosomes released by the same parasite. This could be addressed by purification and characterisation of exosomes in other time points after infection (e.g. 1-, 3- and/or 5 dpi). Previous studies have shown that the early host immune responses determine the establishment of the parasite (Stear *et al.*, 1995, Stear *et al.*, 1999a, Stear *et al.*, 1999b) and that the protein profile of *T. circumcincta*'s ES

products is different among developmental stages (Balic *et al.*, 2003, Smith *et al.*, 2009), which suggests that the proteins included in the exosomes might also be different.

The immunogenicity of the contents of the exosomes was demonstrated in immunoblotting experiments using serum from previously infected and helminth naive sheep. The proteins were bound by IgG and IgA in serum from the previously infected sheep. This confirms that the exosomes might be released *in vivo* by the early parasitic stages of *T. circumcincta*, and that some of the proteins that the exosomes contain might be potential vaccine and/or drug candidates for the future control of *T. circumcincta*. To date, this was the first time that the immunogenicity of parasite derived exosomes was examined. Future studies should examine whether exosomes derived from other parasites are recognised by their hosts' immune responses.

Future relevant studies would include the determination of the source of the *T. circumcincta* exosomes. This could be achieved by performing TEM, immunochemistry-electron microscopy or scanning electron microscopy (SEM). It was shown that the trematodes *F. hepatica* and *E. caproni* release the exosomes from their tegument (Threadgold, 1963, Andresen *et al.*, 1989, Marcilla *et al.*, 2012), whilst in the nematode *H. polygyrus* the exosomes originate from the intestine (Buck *et al.*, 2014). Another potentially useful avenue of research would be to examine the presence and to characterise mRNAs and miRNAs that are present in the exosomes of *T. circumcincta* and compare them with the miRNAs derived from total ES products, if any. It has been suggested that secretion of miRNAs into the host is conserved among the nematodes since, thus far, they have been identified in *H. polygyrus* and *Litomosoides sigmodontis* (Buck *et al.*, 2014). The miRNAs contained in exosomes derived from *H. polygyrus* are manipulating the immune responses of the host by suppressing both the Th2 responses and the eosinophilia (Buck *et al.*, 2014). The identification of the ovine genes whose transcript expression might be regulated by the miRNAs present in the *T. circumcincta* exosomes would enhance our understanding of the host-parasite interactions and the immune evasion. Moreover, it would be useful to see whether the miRNAs regulate any of the *T.*

circumcincta genes, as the exosomes might aid the communication among the parasites. Verification with qPCR that miRNAs can be detected in *T. circumcincta* exosomes would be of great value because this would validate the accuracy of the sequencing results. Finally, another important experiment would be to ascertain whether or not these vesicles are taken up by the host cells *in vivo*, as this would be indicative of a role in the host-parasite interface. This could be shown by taking samples from the abomasum of sheep 1-week post infection and perform immunohistochemistry; or culturing epithelial cells *in vitro*, expose them to labelled exosomes and examine uptake with confocal microscopy. The latter had been done for the *E. caproni* derived exosomes, which led the authors to suggest that exosomes play an important role in host-parasite interactions (Marcilla *et al.*, 2012).

6.1 Summary

In summary, the most important findings of this PhD project are the following:

1) Monitoring the transcript levels of the RNAi pathway genes *Ce-dcr-1, Ce-ego-1* and *Ce-rsd-3* cannot be used as controls to indicate activation of the RNAi pathway in response to exposure to exogenous gene-specific dsRNA in *C. elegans*.

2) *T. circumcincta* expresses 58 genes that encode ASP molecules. Some of the genes that contain a signal peptide in their sequence are expressed in different stages of the parasite. The majority of the genes were detected in L3 after exsheathment with CO_2, but not after exsheathment by NaClO suggesting that the exsheathment protocol had an impact on gene expression.

3) RNAi was successful in *T. circumcincta* after 1 h of soaking in gene-specific dsRNA. The transcript of the target gene recovered to normal levels 48 h after successful RNAi. Inconsistent silencing was observed not only among different genes, but also among different biological replicates of a single target gene.

4) Potential reasons for the inconsistencies were examined and it was found that storage period of the larvae affects the consistency of the RNAi results. Larvae

stored for a short period of time (less than a month) were susceptible, whilst larvae stored for a year were refractory to RNAi.

5) *T. circumcincta* actively releases EMVs. The EMVs contain several unique and novel proteins, some of which were found to be targets of IgA and IgG responses in infected sheep. Some of the proteins found in the EMVs could potentially be useful vaccine candidates for the future control of the parasite.

Bibliography

ALMERIA, S., CANALS, A., ZARLENGA, D. S. & GASBARRE, L. C. 1997. Quantification of cytokine gene expression in lamina propria lymphocytes of cattle following infection with Ostertagia ostertagi. *J Parasitol,* 83**,** 1051-5.

ANDERSEN, J. S. & MANN, M. 2006. Organellar proteomics: turning inventories into insights. *EMBO Rep,* 7**,** 874-9.

ANDERSON, R. C. 2000. Nematode parasites of vertebrates - their development and transmission. 2nd ed. New York, USA: CABI Publishing.

ANDERSON, T. J., BLOUIN, M. S. & BEECH, R. N. 1998. Population biology of parasitic nematodes: applications of genetic markers. *Adv Parasitol,* 41**,** 219-83.

ANDREOLA, G., RIVOLTINI, L., CASTELLI, C., HUBER, V., PEREGO, P., DEHO, P., SQUARCINA, P., ACCORNERO, P., LOZUPONE, F., LUGINI, L., STRINGARO, A., MOLINARI, A., ARANCIA, G., GENTILE, M., PARMIANI, G. & FAIS, S. 2002. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J Exp Med,* 195**,** 1303-16.

ANDRESEN, K., SIMONSEN, P. E., ANDERSEN, B. J. & BIRCH-ANDERSEN, A. 1989. Echinostoma caproni in mice: shedding of antigens from the surface of an intestinal trematode. *Int J Parasitol,* 19**,** 111-8.

ANDREWS, S. J., HOLE, N. J., MUNN, E. A. & ROLPH, T. P. 1995. Vaccination of sheep against haemonchosis with H11, a gut membrane-derived protective antigen from the adult parasite: prevention of the periparturient rise and colostral transfer of protective immunity. *Int J Parasitol,* 25**,** 839-46.

ASOJO, O. A., GOUD, G., DHAR, K., LOUKAS, A., ZHAN, B., DEUMIC, V., LIU, S., BORGSTAHL, G. E. O. & HOTEZ, P. J. 2005. X-ray structure of Na-ASP-2, a pathogenesis-related-1 protein from the nematode parasite, *Necator americanus*, and a vaccine antigen for human hookworm infection. *Journal of Molecular Biology,* 346**,** 801-814.

ATHANASIADOU, S., KYRIAZAKIS, I., JACKSON, F. & COOP, R. L. 2000. Consequences of long-term feeding with condensed tannins on sheep parasitised with Trichostrongylus colubriformis. *Int J Parasitol,* 30**,** 1025-33.

ATHANASIADOU, S. & HUNTLEY, J. F. 2008. Emerging technologies and their applications in interactions between nutrition and immunity to gastrointestinal parasites in sheep. *Parasite Immunol,* 30**,** 101-11.

BAHUAUD, D., MARTINEZ-ORTIZ DE MONTELLANO, C., CHAUVEAU, S., PREVOT, F., TORRES-ACOSTA, F., FOURASTE, I. & HOSTE, H. 2006. Effects of four tanniferous plant extracts on the in vitro exsheathment of third-stage larvae of parasitic nematodes. *Parasitology,* 132**,** 545-54.

BALIC, A., BOWLES, V. M. & MEEUSEN, E. N. 2000a. Cellular profiles in the abomasal mucosa and lymph node during primary infection with Haemonchus contortus in sheep. *Vet Immunol Immunopathol,* 75**,** 109-20.

BALIC, A., BOWLES, V. M. & MEEUSEN, E. N. T. 2000b. The immunobiology of gastrointestinal nematode infections in ruminants. *Advances in Parasitology.* Academic Press.

BALIC, A., BOWLES, V. M., LIU, Y. S. & MEEUSEN, E. N. T. 2003. Local immune responses in sensitized sheep following challenge infection with *Teladorsagia circumcincta*. *Parasite Immunology,* 25**,** 375-381.

BALIC, A., CUNNINGHAM, C. P. & MEEUSEN, E. N. 2006. Eosinophil interactions with Haemonchus contortus larvae in the ovine gastrointestinal tract. *Parasite Immunol,* 28**,** 107-15.

BARGER, I. 1997. Control by management. *Vet Parasitol,* 72**,** 493-500.

BARGER, I. A. 1993. Influence of sex and reproductive status on susceptibility of ruminants to nematode parasitism. *International Journal for Parasitology,* 23**,** 463-469.

BARGER, I. A. 1999. The role of epidemiological knowledge and grazing management for helminth control in small ruminants. *Int J Parasitol,* 29**,** 41-7; discussion 49-50.

BARNES, E. H., DOBSON, R. J. & BARGER, I. A. 1995. Worm control and anthelmintic resistance: adventures with a model. *Parasitol Today,* 11**,** 56-63.

BARTLEY, D. J., JACKSON, E., JOHNSTON, K., COOP, R. L., MITCHELL, G. B., SALES, J. & JACKSON, F. 2003. A survey of anthelmintic resistant nematode parasites in Scottish sheep flocks. *Vet Parasitol,* 117**,** 61-71.

BATYCKA, M., INGLIS, N. F., COOK, K., ADAM, A., FRASER-PITT, D., SMITH, D. G., MAIN, L., LUBBEN, A. & KESSLER, B. M. 2006. Ultra-fast tandem mass spectrometry scanning combined with monolithic column liquid chromatography increases throughput in proteomic analysis. *Rapid Commun Mass Spectrom,* 20**,** 2074-80.

BERALDI, D., CRAIG, B. H., BISHOP, S. C., HOPKINS, J. & PEMBERTON, J. M. 2008. Phenotypic analysis of host–parasite interactions in lambs infected with *Teladorsagia circumcincta*. *International Journal for Parasitology,* 38**,** 1567-1577.

BERNAL, D., TRELIS, M., MONTANER, S., CANTALAPIEDRA, F., GALIANO, A., HACKENBERG, M. & MARCILLA, A. 2014. Surface analysis of Dicrocoelium dendriticum. The molecular characterization of exosomes reveals the presence of miRNAs. *Journal of proteomics,* 105**,** 232-41.

BERNSTEIN, E., CAUDY, A. A., HAMMOND, S. M. & HANNON, G. J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature,* 409**,** 363-366.

BESIER, B. 2007. New anthelmintics for livestock: the time is right. *Trends in parasitology,* 23**,** 21-24.

BETHONY, J., LOUKAS, A., SMOUT, M., BROOKER, S., MENDEZ, S., PLIESKATT, J., GOUD, G., BOTTAZZI, M. E., ZHAN, B., WANG, Y., WILLIAMSON, A., LUSTIGMAN, S., CORREA-OLIVEIRA, R., XIAO, S. & HOTEZ, P. J. 2005. Antibodies against a secreted protein from hookworm larvae reduce the intensity of hookworm infection in humans and vaccinated laboratory animals. *FASEB J,* 19**,** 1743-5.

BIN, Z., HAWDON, J., QIANG, S., HAINAN, R., HUIQING, Q., WEI, H., SHU-HUA, X., TIEHUA, L., XING, G., ZHENG, F. & HOTEZ, P. 1999. Ancylostoma secreted protein 1 (ASP-1) homologues in human hookworms. *Mol Biochem Parasitol,* 98**,** 143-9.

BLAXTER, M. L., DE LEY, P., GAREY, J. R., LIU, L. X., SCHELDEMAN, P., VIERSTRAETE, A., VANFLETEREN, J. R., MACKEY, L. Y., DORRIS, M., FRISSE, L. M., VIDA, J. T. & THOMAS, W. K. 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature,* 392**,** 71-5.

BLOUIN, M. S., YOWELL, C. A., COURTNEY, C. H. & DAME, J. B. 1995. Host movement and the genetic structure of populations of parasitic nematodes. *Genetics,* 141**,** 1007-14.

BOBRIE, A., COLOMBO, M., RAPOSO, G. & THÉRY, C. 2011. Exosome Secretion: Molecular Mechanisms and Roles in Immune Responses. *Traffic,* 12**,** 1659-1668.

BORGERS, M. & DE NOLLIN, S. 1975. Ultrastructural changes in Ascaris suum intestine after mebendazole treatment in vivo. *J Parasitol,* 61**,** 110-22.

BOUIX, J., KRUPINSKI, J., RZEPECKI, R., NOWOSAD, B., SKRZYZALA, I., ROBORZYNSKI, M., FUDALEWICZ-NIEMCZYK, W., SKALSKA, M., MALCZEWSKI, A. & GRUNER, L. 1998. Genetic resistance to gastrointestinal nematode parasites in Polish long-wool sheep. *Int J Parasitol,* 28**,** 1797-804.

BOYLE, J. P., WU, X. J., SHOEMAKER, C. B. & YOSHINO, T. P. 2003. Using RNA interference to manipulate endogenous gene expression in Schistosoma mansoni sporocysts. *Mol Biochem Parasitol,* 128**,** 205-15.

BRENNER, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics,* 77**,** 71-94.

BRITTON, C. & MURRAY, L. 2006. Using *Caenorhabditis elegans* for functional analysis of genes of parasitic nematodes. *International Journal for Parasitology,* 36**,** 651-659.

BRUNET, S., AUFRERE, J., EL BABILI, F., FOURASTE, I. & HOSTE, H. 2007. The kinetics of exsheathment of infective nematode larvae is disturbed in the presence of a tannin-rich plant extract (sainfoin) both in vitro and in vivo. *Parasitology,* 134**,** 1253-62.

BRUNET, S., JACKSON, F. & HOSTE, H. 2008. Effects of sainfoin (Onobrychis viciifolia) extract and monomers of condensed tannins on the association of abomasal nematode larvae with fundic explants. *International journal for parasitology,* 38**,** 783-90.

BRUNET, S., FOURQUAUX, I. & HOSTE, H. 2011. Ultrastructural changes in the third-stage, infective larvae of ruminant nematodes treated with sainfoin (Onobrychis viciifolia) extract. *Parasitol Int,* 60**,** 419-24.

BRUNSDON, R. V. 1980. Principles of helminth control. *Veterinary Parasitology,* 6**,** 185-215.

BUCK, A., COAKLEY, G., SIMBARI, F., MCSORLEY, H. J., QUINTANA, J., LE BIHAN, T., KUMAR, S., ABREU-GOODGER, C., LEAR, M., HARCUS, Y., CERONI, A., BABAYAN, S. A., BLAXTER, M. L., IVENS, I. & MAIZELS, R. M. 2014. Exosomes secreted by a nematode parasite transfer small RNAs to mammalian cells and regulate genes of the innate immune system. *Nature Communications***,** in press.

BURGESS, C. G., BARTLEY, Y., REDMAN, E., SKUCE, P. J., NATH, M., WHITELAW, F., TAIT, A., GILLEARD, J. S. & JACKSON, F. 2012. A survey of the trichostrongylid nematode species present on UK sheep farms and associated anthelmintic control practices. *Vet Parasitol,* 189**,** 299-307.

CANALS, A. & GASBARRE, L. C. 1990. Ostertagia ostertagi: isolation and partial characterization of somatic and metabolic antigens. *Int J Parasitol,* 20**,** 1047-54.

CANTACESSI, C., CAMPBELL, B. E., VISSER, A., GELDHOF, P., NOLAN, M. J., NISBET, A. J., MATTHEWS, J. B., LOUKAS, A., HOFMANN, A., OTRANTO, D., STERNBERG, P. W. & GASSER, R. B. 2009. A portrait of the "SCP/TAPS" proteins of eukaryotes — Developing a framework for fundamental research and biotechnological outcomes. *Biotechnology Advances,* 27**,** 376-388.

CARLIER, M. F., PERNIER, J. & AVVARU, B. S. 2013. Control of actin filament dynamics at barbed ends by WH2 domains: from capping to permissive and processive assembly. *Cytoskeleton (Hoboken),* 70**,** 540-9.

CAUDY, A. A., KETTING, R. F., HAMMOND, S. M., DENLI, A. M., BATHOORN, A. M. P., TOPS, B. B. J., SILVA, J. M., MYERS, M. M., HANNON, G. J. & PLASTERK, R. H. A. 2003. A micrococcal nuclease homologue in RNAi effector complexes. *Nature,* 425**,** 411-414.

CHALMERS, I. W., MCARDLE, A. J., COULSON, R. M., WAGNER, M. A., SCHMID, R., HIRAI, H. & HOFFMANN, K. F. 2008. Developmentally regulated expression, alternative splicing and distinct sub-groupings in members of the Schistosoma mansoni venom allergen-like (SmVAL) gene family. *BMC Genomics,* 9**,** 89.

CHALMERS, I. W. & HOFFMANN, K. F. 2012. Platyhelminth Venom Allergen-Like (VAL) proteins: revealing structural diversity, class-specific features and biological associations across the phylum. *Parasitology,* 139**,** 1231-1245.

CHANDRAWATHANI, P., JAMNAH, O., WALLER, P. J., HOGLUND, J., LARSEN, M. & ZAHARI, W. M. 2002. Nematophagous fungi as a biological control agent for nematode parasites of small ruminants in Malaysia: a special emphasis on Duddingtonia flagrans. *Vet Res,* 33**,** 685-96.

CHAPMAN, C. B. & MITCHELL, G. F. 1982. Proteolytic cleavage of immunoglobulin by enzymes released by Fasciola hepatica. *Veterinary parasitology,* 11**,** 165-78.

CHARLES, T. P., ROQUE, M. V. & SANTOS, C. D. 1996. Reduction of Haemonchus contortus infective larvae by Harposporium anguillulae in sheep faecal cultures. *Int J Parasitol,* 26**,** 509-10.

CHARLESTON, W. A. G. 1965. Pathogenesis of experimental haemonchosis in sheep, with special reference to the development of resistance. *Journal of Comparative Pathology,* 75**,** 55-67.

CHARTIER, C. & PORS, I. 2003. Effect of the nematophagous fungus, Duddingtonia flagrans, on the larval development of goat parasitic nematodes: a plot study. *Vet Res,* 34**,** 221-30.

CLAEREBOUT, E., AGNEESSENS, SHAW, D. J. & VERCRUYSSE, J. 1999. Larval migration inhibition activity in abomasal mucus and serum from calves infected with Ostertagia ostertagi. *Res Vet Sci,* 66**,** 253-7.

COCUCCI, E., RACCHETTI, G. & MELDOLESI, J. 2009. Shedding microvesicles: artefacts no more. *Trends Cell Biol,* 19**,** 43-51.

COLES, G. C. 1986. Anthelmintic resistance in sheep. *Vet Clin North Am Food Anim Pract,* 2**,** 423-32.

CONDER, G. A. & JOHNSON, S. S. 1996. Viability of infective larvae of *Haemonchus contortus*, *Ostertagia ostertagi*, and *Trichostrongylus colubriformis* following exsheathment by various techniques. *Journal of Parasitology,* 82**,** 100-102.

COOP, R. L. & KYRIAZAKIS, I. 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends Parasitol,* 17**,** 325-30.

CORRENTI, J. M. & PEARCE, E. J. 2004. Transgene expression in *Schistosoma mansoni*: introduction of RNA into schistosomula by electroporation. *Molecular and Biochemical Parasitology,* 137**,** 75-79.

COUZIN, J. 2005. The Ins and Outs of Exosomes. *Science,* 308**,** 1862-1863.

CRAIG, H., WASTLING, J. M. & KNOX, D. P. 2006. A preliminary proteomic survey of the in vitro excretory/secretory products of fourth-stage larval and adult *Teladorsagia circumcincta*. *Parasitology,* 132**,** 535-543.

CRAIG, N. M., MILLER, H. R., SMITH, W. D. & KNIGHT, P. A. 2007. Cytokine expression in naive and previously infected lambs after challenge with Teladorsagia circumcincta. *Vet Immunol Immunopathol,* 120**,** 47-54.

CRAIG, N. M., SMITH, D. W., PATE, J. A., MORRISON, I. W. & KNIGHT, P. A. 2014. Local cytokine transcription in naive and previously infected sheep and lambs following challenge with Teladorsagia circumcincta. *BMC Vet Res,* 10**,** 87.

CROFTON, H. D. & WHITLOCK, J. H. 1965. Ecology and biological plasticity of sheep nematodes. III. Studies on Ostertagia circumcincta (Stadelmann, 1894). *Cornell Veterinarian,* 55**,** 259-62.

CRUZ-ROJO, M. A., MARTINEZ-VALLADARES, M. & ROJO-VAZQUEZ, F. A. 2012. Teladorsagia circumcincta antibodies in serum and milk samples in experimentally infected lactating ewes. *Vet Parasitol,* 188**,** 386-90.

DALTON, J. P. & HEFFERNAN, M. 1989. Thiol proteases released in vitro by Fasciola hepatica. *Molecular and biochemical parasitology,* 35**,** 161-6.

DALTON, J. P., MCGONIGLE, S., ROLPH, T. P. & ANDREWS, S. J. 1996. Induction of protective immunity in cattle against infection with Fasciola hepatica by vaccination with cathepsin L proteinases and with hemoglobin. *Infection and immunity,* 64**,** 5066-74.

DALTON, J. P., ROBINSON, M. W., MULCAHY, G., O'NEILL, S. M. & DONNELLY, S. 2013. Immunomodulatory molecules of Fasciola hepatica: candidates for both vaccine and immunotherapeutic development. *Veterinary parasitology,* 195**,** 272-85.

DALZELL, J. J., MCVEIGH, P., WARNOCK, N. D., MITREVA, M., BIRD, D. M., ABAD, P., FLEMING, C. C., DAY, T. A., MOUSLEY, A., MARKS, N. J. & MAULE, A. G. 2011. RNAi Effector Diversity in Nematodes. *Plos Neglected Tropical Diseases,* 5**,** e1176.

DATU, B. J., GASSER, R. B., NAGARAJ, S. H., ONG, E. K., O'DONOGHUE, P., MCINNES, R., RANGANATHAN, S. & LOUKAS, A. 2008. Transcriptional changes in the hookworm, Ancylostoma caninum, during the transition from a free-living to a parasitic larva. *PLoS Negl Trop Dis,* 2**,** e130.

DAVIS, M. W., SOMERVILLE, D., LEE, R. Y., LOCKERY, S., AVERY, L. & FAMBROUGH, D. M. 1995. Mutations in the Caenorhabditis elegans Na,K-

ATPase alpha-subunit gene, eat-6, disrupt excitable cell function. *J Neurosci,* 15**,** 8408-18.

DAWKINS, H. J., WINDON, R. G. & EAGLESON, G. K. 1989. Eosinophil responses in sheep selected for high and low responsiveness to Trichostrongylus colubriformis. *Int J Parasitol,* 19**,** 199-205.

DE MAERE, V., VERCAUTEREN, I., SAVERWYNS, H., CLAEREBOUT, E., BERX, G. & VERCRUYSSE, J. 2002. Identification of potential protective antigens of Ostertagia ostertagi with local antibody probes. *Parasitology,* 125**,** 383-91.

DENHAM, D. A. 1969. The development of *Ostertagia circumcincta* in lambs. *Journal of Helminthology,* 43**,** 299-310.

DENT, J. A., SMITH, M. M., VASSILATIS, D. K. & AVERY, L. 2000. The genetics of ivermectin resistance in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 2674-9.

DOMEIER, M. E., MORSE, D. P., KNIGHT, S. W., PORTEREIKO, M., BASS, B. L. & MANGO, S. E. 2000. A link between RNA interference and nonsense-mediated decay in Caenorhabditis elegans. *Science,* 289**,** 1928-31.

DONALDSON, J., VAN HOUTERT, M. F. J. & SYKES, A. R. 1998. The effect of nutrition on the periparturient parasite status of mature ewes. *Animal Science,* 67**,** 523-533.

DOUCH, P. G., HARRISON, G. B., BUCHANAN, L. L. & GREER, K. S. 1983. In vitro bioassay of sheep gastrointestinal mucus for nematode paralysing activity mediated by substances with some properties characteristic of SRS-A. *Int J Parasitol,* 13**,** 207-12.

DRUDGE, J. H., LELAND, S. E., JR. & WYANT, Z. N. 1957. Strain variation in the response of sheep nematodes to the action of phenothiazine. II. Studies on pure infections of Haemonchus contortus. *Am J Vet Res,* 18**,** 317-25.

DRUDGE, J. H. & ELAM, G. 1961. Preliminary observations on the resistance of horse strongyles to phenothiazine. *Journal of Parasitology,* 47**,** 38-39.

DUBUCQUOI, S., DESREUMAUX, P., JANIN, A., KLEIN, O., GOLDMAN, M., TAVERNIER, J., CAPRON, A. & CAPRON, M. 1994. Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *J Exp Med,* 179**,** 703-8.

DUCHAINE, T. F., WOHLSCHLEGEL, J. A., KENNEDY, S., BEI, Y., CONTE, D., JR., PANG, K., BROWNELL, D. R., HARDING, S., MITANI, S., RUVKUN, G., YATES, J. R., 3RD & MELLO, C. C. 2006. Functional proteomics reveals the biochemical niche of C. elegans DCR-1 in multiple small-RNA-mediated pathways. *Cell,* 124**,** 343-54.

DUXBURY, M. S. & WHANG, E. E. 2004. RNA interference: a practical approach. *Journal of Surgical Research,* 117**,** 339-344.

EGER, A., KIRCH, A., MANFRAS, B., KERN, P., SCHULZ-KEY, H. & SOBOSLAY, P. T. 2003. Pro-inflammatory (IL-1beta, IL-18) cytokines and IL-8 chemokine release by PBMC in response to Echinococcus multilocularis metacestode vesicles. *Parasite immunology,* 25**,** 103-5.

EMERY, D. L. 1996. Vaccination against worm parasites of animals. *Vet Parasitol,* 64**,** 31-45.

EPE, C., HOLST, C., KOOPMANN, R., SCHNIEDER, T., LARSEN, M. & VON SAMSON-HIMMELSTJERNA, G. 2009. Experiences with Duddingtonia flagrans administration to parasitized small ruminants. *Vet Parasitol,* 159**,** 86-90.

EPE, C. & KAMINSKY, R. 2013. New advancement in anthelmintic drugs in veterinary medicine. *Trends Parasitol,* 29**,** 129-34.

EVANS, A. M. & MARTIN, R. J. 1996. Activation and cooperative multi-ion block of single nicotinic-acetylcholine channel currents of Ascaris muscle by the tetrahydropyrimidine anthelmintic, morantel. *Br J Pharmacol,* 118**,** 1127-40.

FEINBERG, E. H. & HUNTER, C. P. 2003. Transport of dsRNA into Cells by the Transmembrane Protein SID-1. *Science,* 301**,** 1545-1547.

FELIX, M. A., ASHE, A., PIFFARETTI, J., WU, G., NUEZ, I., BELICARD, T., JIANG, Y., ZHAO, G., FRANZ, C. J., GOLDSTEIN, L. D., SANROMAN, M., MISKA, E. A. & WANG, D. 2011. Natural and experimental infection of Caenorhabditis nematodes by novel viruses related to nodaviruses. *PLoS Biology,* 9**,** e1000586.

FERNANDEZ, C., SZYPERSKI, T., BRUYERE, T., RAMAGE, P., MOSINGER, E. & WUTHRICH, K. 1997. NMR solution structure of the pathogenesis-related protein P14a. *J Mol Biol,* 266**,** 576-93.

FIRE, A., XU, S. Q., MONTGOMERY, M. K., KOSTAS, S. A., DRIVER, S. E. & MELLO, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature,* 391**,** 806-811.

FISCHER, S. E. J. 2010. Small RNA-mediated gene silencing pathways in *C. elegans*. *The International Journal of Biochemistry & Cell Biology,* 42**,** 1306-1315.

FOX, M. T., GERRELLI, D., PITT, S. R., JACOBS, D. E., GILL, M. & GALE, D. L. 1989. *Ostertagia ostertagi* infection in the calf: effects of a trickle challenge on appetite, digestibility, rate of passage of digesta and liveweight gain. *Research in Veterinary Science,* 47**,** 294-298.

FOX, M. T. 1993. Pathophysiology of infection with *Ostertagia ostertagi* in cattle. *Veterinary Parasitology,* 46**,** 143-158.

FOX, M. T. 1997. Pathophysiology of infection with gastrointestinal nematodes in domestic ruminants: recent developments. *Veterinary Parasitology,* 72**,** 285-308.

GEARY, T. G., SIMS, S. M., THOMAS, E. M., VANOVER, L., DAVIS, J. P., WINTERROWD, C. A., KLEIN, R. D., HO, N. F. & THOMPSON, D. P. 1993. Haemonchus contortus: ivermectin-induced paralysis of the pharynx. *Exp Parasitol,* 77**,** 88-96.

GELDHOF, P., CLAEREBOUT, E., KNOX, D., VERCAUTEREN, I., LOOSZOVA, A. & VERCRUYSSE, J. 2002. Vaccination of calves against *Ostertagia ostertagi* with cysteine proteinase enriched protein fractions. *Parasite Immunology,* 24**,** 263-270.

GELDHOF, P., VERCAUTEREN, I., GEVAERT, K., STAES, A., KNOX, D. P., VANDEKERCKHOVE, J., VERCRUYSSE, J. & CLAEREBOUT, E. 2003. Activation-associated secreted proteins are the most abundant antigens in a host protective fraction from *Ostertagia ostertagi*. *Molecular and Biochemical Parasitology,* 128**,** 111-114.

GELDHOF, P., VERCAUTEREN, I., VERCRUYSSE, J., KNOX, D. P., VAN DEN BROECK, W. & CLAEREBOUT, E. 2004. Validation of the protective Ostertagia ostertagi ES-thiol antigens with different adjuvantia. *Parasite Immunol,* 26**,** 37-43.

GELDHOF, P., MOLLOY, C. & KNOX, D. P. 2006a. Combinatorial RNAi on intestinal cathepsin B-like proteinases in *Caenorhabditis elegans* questions the perception of their role in nematode biology. *Molecular and Biochemical Parasitology,* 145**,** 128-132.

GELDHOF, P., MURRAY, L., COUTHIER, A., GILLEARD, J. S., MCLAUCHLAN, G., KNOX, D. P. & BRITTON, C. 2006b. Testing the efficacy of RNA interference in *Haemonchus contortus*. *International Journal for Parasitology,* 36**,** 801-810.

GELDHOF, P., VISSER, A., CLARK, D., SAUNDERS, G., BRITTON, C., GILLEARD, J., BERRIMAN, M. & KNOX, D. 2007. RNA interference in parasitic helminths: current situation, potential pitfalls and future prospects. *Parasitology,* 134**,** 609-619.

GELDHOF, P., MEYVIS, Y., VERCRUYSSE, J. & CLAEREBOUT, E. 2008. Vaccine testing of a recombinant activation-associated secreted protein (ASP1) from Ostertagia ostertagi. *Parasite Immunol,* 30**,** 57-60.

GHOSH, K., HAWDON, J. & HOTEZ, P. 1996. Vaccination with alum-precipitated recombinant Ancylostoma-secreted protein 1 protects mice against challenge infections with infective hookworm (Ancylostoma caninum) larvae. *J Infect Dis,* 174**,** 1380-3.

GHOSH, K. & HOTEZ, P. J. 1999. Antibody-dependent reductions in mouse hookworm burden after vaccination with *Ancylostoma caninum* secreted protein 1. *Journal of Infectious Diseases,* 180**,** 1674-1681.

GIBBS, H. C. 1986. Hypobiosis in parasitic nematodes--an update. *Advances in parasitology,* 25**,** 129-74.

GIBSON, U. E., HEID, C. A. & WILLIAMS, P. M. 1996. A novel method for real time quantitative RT-PCR. *Genome Research,* 6**,** 995-1001.

GILL, H. S., COLDITZ, I. G. & WATSON, D. L. 1993a. Immune responsiveness of lambs selected for resistance to haemonchosis. *Res Vet Sci,* 54**,** 361-5.

GILL, H. S., GRAY, G. D., WATSON, D. L. & HUSBAND, A. J. 1993b. Isotype-specific antibody responses to Haemonchus contortus in genetically resistant sheep. *Parasite Immunol,* 15**,** 61-7.

GILL, H. S. 1994. Cell-mediated immunity in Merino lambs with genetic resistance to Haemonchus contortus. *Int J Parasitol,* 24**,** 749-56.

GOUD, G. N., ZHAN, B., GHOSH, K., LOUKAS, A., HAWDON, J., DOBARDZIC, A., DEUMIC, V., LIU, S., DOBARDZIC, R., ZOOK, B. C., JIN, Q., LIU, Y. Y., HOFFMAN, L., CHUNG-DEBOSE, S., PATEL, R., MENDEZ, S. & HOTEZ, P. J. 2004. Cloning, yeast expression, isolation, and vaccine testing of recombinant Ancylostoma-secreted protein (ASP)-1 and ASP-2 from *Ancylostoma ceylanicum*. *Journal of Infectious Diseases,* 189**,** 919-929.

GRETES, M. C., POOLE, L. B. & KARPLUS, P. A. 2012. Peroxiredoxins in parasites. *Antioxidants & redox signaling,* 17**,** 608-33.

GRISHOK, A. 2005. RNAi mechanisms in *Caenorhabditis elegans*. *FEBS Letters,* 579**,** 5932-5939.

GRONVOLD, J., WOLSTRUP, J., LARSEN, M., HENRIKSEN, S. A. & NANSEN, P. 1993. Biological control of Ostertagia ostertagi by feeding selected nematode-trapping fungi to calves. *J Helminthol,* 67**,** 31-6.

GUANG, S., BOCHNER, A. F., PAVELEC, D. M., BURKHART, K. B., HARDING, S., LACHOWIEC, J. & KENNEDY, S. 2008. An Argonaute transports siRNAs from the cytoplasm to the nucleus. *Science,* 321**,** 537-41.

GUO, S. & KEMPHUES, K. J. 1995. par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell,* 81**,** 611-620.

HALLIDAY, A. M., ROUTLEDGE, C. M., SMITH, S. K., MATTHEWS, J. B. & SMITH, W. D. 2007. Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep. *Parasite Immunology,* 29**,** 425-434.

HALLIDAY, A. M., MORRISON, W. I. & SMITH, W. D. 2009. Kinetics of the local cellular response in the gastric lymph of immune and susceptible sheep to infection with *Teladorsagia circumcincta*. *Parasite Immunology,* 31**,** 402-411.

HALLIDAY, A. M., LAINSON, F. A., YAGA, R., INGLIS, N. F., BRIDGETT, S., NATH, M. & KNOX, D. P. 2012. Transcriptional changes in *Teladorsagia circumcincta* upon encountering host tissue of differing immune status. *Parasitology,* 139**,** 387-405.

HAMMOND, S. M., BERNSTEIN, E., BEACH, D. & HANNON, G. J. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature,* 404**,** 293-296.

HAMMOND, S. M. 2005. Dicing and slicing: The core machinery of the RNA interference pathway. *FEBS Letters,* 579**,** 5822-5829.

HAWDON, J. M., JONES, B. F., HOFFMAN, D. R. & HOTEZ, P. J. 1996. Cloning and characterization of Ancylostoma-secreted protein - A novel protein associated with the transition to parasitism by infective hookworm larvae. *Journal of Biological Chemistry,* 271**,** 6672-6678.

HAWDON, J. M., NARASIMHAN, S. & HOTEZ, P. J. 1999. Ancylostoma secreted protein 2: cloning and characterization of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*. *Molecular and Biochemical Parasitology,* 99**,** 149-165.

HEID, C. A., STEVENS, J., LIVAK, K. J. & WILLIAMS, P. M. 1996. Real time quantitative PCR. *Genome Research,* 6**,** 986-994.

HEWITSON, J. P., HARCUS, Y., MURRAY, J., VAN AGTMAAL, M., FILBEY, K. J., GRAINGER, J. R., BRIDGETT, S., BLAXTER, M. L., ASHTON, P. D., ASHFORD, D. A., CURWEN, R. S., WILSON, R. A., DOWLE, A. A. & MAIZELS, R. M. 2011. Proteomic analysis of secretory products from the model gastrointestinal nematode Heligmosomoides polygyrus reveals dominance of venom allergen-like (VAL) proteins. *J Proteomics,* 74**,** 1573-94.

HOOGEWIJS, D., HOUTHOOFD, K., MATTHIJSSENS, F., VANDESOMPELE, J. & VANFLETEREN, J. R. 2008. Selection and validation of a set of reliable

reference genes for quantitative sod gene expression analysis in *C. elegans*. *Bmc Molecular Biology,* 9.

HOSTE, H., CHARTIER, C. & LE FRILEUX, Y. 2002. Control of gastrointestinal parasitism with nematodes in dairy goats by treating the host category at risk. *Vet Res,* 33**,** 531-45.

HOSTE, H., MARTINEZ-ORTIZ-DE-MONTELLANO, C., MANOLARAKI, F., BRUNET, S., OJEDA-ROBERTOS, N., FOURQUAUX, I., TORRES-ACOSTA, J. F. & SANDOVAL-CASTRO, C. A. 2012. Direct and indirect effects of bioactive tannin-rich tropical and temperate legumes against nematode infections. *Veterinary parasitology,* 186**,** 18-27.

HOTSON, I. K. 1982. The avermectins: A new family of antiparasitic agents. *J S Afr Vet Assoc,* 53**,** 87-90.

HOUDIJK, J. G., KYRIAZAKIS, I., JACKSON, F., HUNTLEY, J. F. & COOP, R. L. 2000. Can an increased intake of metabolizable protein affect the periparturient relaxation in immunity against teladorsagia circumcincta in sheep? *Vet Parasitol,* 91**,** 43-62.

HOUDIJK, J. G. M., KYRIAZAKIS, I., JACKSON, F. & COOP, R. L. 2001. The relationship between protein nutrition, reproductive effort and breakdown in immunity to Teladorsagia circumcincta in periparturient ewes. *Animal Science,* 72**,** 595-606.

HRISTOV, M., ERL, W., LINDER, S. & WEBER, P. C. 2004. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. *Blood,* 104**,** 2761-6.

HSU, C., MOROHASHI, Y., YOSHIMURA, S., MANRIQUE-HOYOS, N., JUNG, S., LAUTERBACH, M. A., BAKHTI, M., GRONBORG, M., MOBIUS, W., RHEE, J., BARR, F. A. & SIMONS, M. 2010. Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. *J Cell Biol,* 189**,** 223-32.

HUNTLEY, J. F., SCHALLIG, H. D., KOOYMAN, F. N., MACKELLAR, A., JACKSON, F. & SMITH, W. D. 1998a. IgE antibody during infection with the ovine abomasal nematode, Teladorsagia circumcincta: primary and secondary responses in serum and gastric lymph of sheep. *Parasite Immunol,* 20**,** 565-71.

HUNTLEY, J. F., SCHALLIG, H. D., KOOYMAN, F. N., MACKELLAR, A., MILLERSHIP, J. & SMITH, W. D. 1998b. IgE responses in the serum and gastric lymph of sheep infected with Teladorsagia circumcincta. *Parasite Immunol,* 20**,** 163-8.

HUNTLEY, J. F., JACKSON, F., COOP, R. L., MACALDOWIE, C., HOUDIJK, J. G., FAMILTON, A. S., XIEH, H. L., STANKIEWICZ, M. & SYKES, A. R. 2004. The sequential analysis of local inflammatory cells during abomasal nematode infection in periparturient sheep. *Veterinary immunology and immunopathology,* 97**,** 163-76.

HUSSEIN, A. S., KICHENIN, K. & SELKIRK, M. E. 2002. Suppression of secreted acetylcholinesterase expression in *Nippostrongylus brasiliensis* by RNA interference. *Molecular and Biochemical Parasitology,* 122**,** 91-94.

HUTVAGNER, G., MCLACHLAN, J., PASQUINELLI, A. E., BALINT, E., TUSCHL, T. & ZAMORE, P. D. 2001. A cellular function for the RNA-

interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science,* 293**,** 834-838.

ISSA, Z., GRANT, W. N., STASIUK, S. & SHOEMAKER, C. B. 2005. Development of methods for RNA interference in the sheep gastrointestinal parasite, *Trichostrongylus colubriformis*. *International Journal for Parasitology,* 35**,** 935-940.

JACKSON, F. & COOP, R. L. 2000. The development of anthelmintic resistance in sheep nematodes. *Parasitology,* 120 Suppl**,** S95-107.

JALKANEN, J., HUHTANIEMI, I. & POUTANEN, M. 2005. Mouse cysteine-rich secretory protein 4 (CRISP4): a member of the Crisp family exclusively expressed in the epididymis in an androgen-dependent manner. *Biol Reprod,* 72**,** 1268-74.

JARRETT, W. F., JENNINGS, F. W., MC, I. W., MULLIGAN, W. & URQUHART, G. M. 1958. Irradiated helminth larvae in vaccination. *Proc R Soc Med,* 51**,** 743-4.

JARRETT, W. F., JENNINGS, F. W., MC, I. W., MULLIGAN, W. & SHARP, N. C. 1961. Studies on immunity to Haemonchus contortus infection--double vaccination of sheep with irradiated larvae. *Am J Vet Res,* 22**,** 186-8.

JASMER, D. P., PERRYMAN, L. E., CONDER, G. A., CROW, S. & MCGUIRE, T. 1993. Protective immunity to Haemonchus contortus induced by immunoaffinity isolated antigens that share a phylogenetically conserved carbohydrate gut surface epitope. *J Immunol,* 151**,** 5450-60.

KAHN, L. P., KNOX, M. R., GRAY, G. D., LEA, J. M. & WALKDEN-BROWN, S. W. 2003. Enhancing immunity to nematode parasites in single-bearing Merino ewes through nutrition and genetic selection. *Vet Parasitol,* 112**,** 211-25.

KAMATH, R. S., FRASER, A. G., DONG, Y., POULIN, G., DURBIN, R., GOTTA, M., KANAPIN, A., LE BOT, N., MORENO, S., SOHRMANN, M., WELCHMAN, D. P., ZIPPERLEN, P. & AHRINGER, J. 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature,* 421**,** 231-237.

KAMINSKY, R., DUCRAY, P., JUNG, M., CLOVER, R., RUFENER, L., BOUVIER, J., WEBER, S. S., WENGER, A., WIELAND-BERGHAUSEN, S., GOEBEL, T., GAUVRY, N., PAUTRAT, F., SKRIPSKY, T., FROELICH, O., KOMOIN-OKA, C., WESTLUND, B., SLUDER, A. & MASER, P. 2008a. A new class of anthelmintics effective against drug-resistant nematodes. *Nature,* 452**,** 176-180.

KAMINSKY, R., GAUVRY, N., WEBER, S. S., SKRIPSKY, T., BOUVIER, J., WENGER, A., SCHROEDER, F., DESAULES, Y., HOTZ, R., GOEBEL, T., HOSKING, B. C., PAUTRAT, F., WIELAND-BERGHAUSEN, S. & DUCRAY, P. 2008b. Identification of the amino-acetonitrile derivative monepantel (AAD 1566) as a new anthelmintic drug development candidate. *Parasitology Research,* 103**,** 931-939.

KAPLAN, R. M. 2002. Anthelmintic resistance in nematodes of horses. *Vet Res,* 33**,** 491-507.

KAPLAN, R. M. 2004. Drug resistance in nematodes of veterinary importance: a status report. *Trends in parasitology,* 20**,** 477-481.

KASAHARA, M., GUTKNECHT, J., BREW, K., SPURR, N. & GOODFELLOW, P. N. 1989. Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. *Genomics,* 5**,** 527-34.

KENNEDY, S., WANG, D. & RUVKUN, G. 2004. A conserved siRNA-degrading RNase negatively regulates RNA interference in C. elegans. *Nature,* 427**,** 645-9.

KENYON, F. & JACKSON, F. 2012. Targeted flock/herd and individual ruminant treatment approaches. *Vet Parasitol,* 186**,** 10-7.

KERBOEUF, D., BLACKHALL, W., KAMINSKY, R. & VON SAMSON-HIMMELSTJERNA, G. 2003. P-glycoprotein in helminths: function and perspectives for anthelmintic treatment and reversal of resistance. *Int J Antimicrob Agents,* 22**,** 332-46.

KETTING, R. F., FISCHER, S. E. J., BERNSTEIN, E., SIJEN, T., HANNON, G. J. & PLASTERK, R. H. A. 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes & Development,* 15**,** 2654-2659.

KNIGHT, S. W. & BASS, B. L. 2001. A Role for the RNase III Enzyme DCR-1 in RNA Interference and Germ Line Development in *Caenorhabditis elegans*. *Science,* 293**,** 2269-2271.

KNOX, D. P. & JONES, D. G. 1990. Studies on the presence and release of proteolytic enzymes (proteinases) in gastro-intestinal nematodes of ruminants. *International journal for parasitology,* 20**,** 243-9.

KNOX, D. P., SMITH, S. K. & SMITH, W. D. 1999. Immunization with an affinity purified protein extract from the adult parasite protects lambs against infection with Haemonchus contortus. *Parasite Immunol,* 21**,** 201-10.

KNOX, D. P. 2000. Development of vaccines against gastrointestinal nematodes. *Parasitology,* 120**,** 43-61.

KNOX, D. P., REDMOND, D. L., SKUCE, P. J. & NEWLANDS, G. F. 2001. The contribution of molecular biology to the development of vaccines against nematode and trematode parasites of domestic ruminants. *Vet Parasitol,* 101**,** 311-35.

KNOX, D. P., GELDHOF, P., VISSER, A. & BRITTON, C. 2007. RNA interference in parasitic nematodes of animals: a reality check? *Trends in parasitology,* 23**,** 105-107.

KOHLER, P. & BACHMANN, R. 1981. Intestinal tubulin as possible target for the chemotherapeutic action of mebendazole in parasitic nematodes. *Mol Biochem Parasitol,* 4**,** 325-36.

KOHLER, P. 2001. The biochemical basis of anthelmintic action and resistance. *Int J Parasitol,* 31**,** 336-45.

KOOYMAN, F. N., VAN KOOTEN, P. J., HUNTLEY, J. F., MACKELLAR, A., CORNELISSEN, A. W. & SCHALLIG, H. D. 1997. Production of a monoclonal antibody specific for ovine immunoglobulin E and its application to monitor serum IgE responses to Haemonchus contortus infection. *Parasitology,* 114 (Pt 4)**,** 395-406.

KOOYMAN, F. N., SCHALLIG, H. D., VAN LEEUWEN, M. A., MACKELLAR, A., HUNTLEY, J. F., CORNELISSEN, A. W. & VERVELDE, L. 2000. Protection in lambs vaccinated with Haemonchus contortus antigens is age

related, and correlates with IgE rather than IgG1 antibody. *Parasite Immunol,* 22**,** 13-20.

KWA, M. S., VEENSTRA, J. G., VAN DIJK, M. & ROOS, M. H. 1995. Beta-tubulin genes from the parasitic nematode Haemonchus contortus modulate drug resistance in Caenorhabditis elegans. *Journal of molecular biology,* 246**,** 500-10.

LACEY, E. 1988. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *Int J Parasitol,* 18**,** 885-936.

LAING, R., KIKUCHI, T., MARTINELLI, A., TSAI, I., BEECH, R., REDMAN, E., HOLROYD, N., BARTLEY, D., BEASLEY, H., BRITTON, C., CURRAN, D., DEVANEY, E., GILABERT, A., HUNT, M., JACKSON, F., JOHNSTON, S., KRYUKOV, I., LI, K., MORRISON, A., REID, A., SARGISON, N., SAUNDERS, G., WASMUTH, J., WOLSTENHOLME, A., BERRIMAN, M., GILLEARD, J. & COTTON, J. 2013. The genome and transcriptome of *Haemonchus contortus*, a key model parasite for drug and vaccine discovery. *Genome Biology,* 14**,** R88.

LANDMANN, F., FOSTER, J. M., SLATKO, B. E. & SULLIVAN, W. 2012. Efficient in vitro RNA interference and immunofluorescence-based phenotype analysis in a human parasitic nematode, Brugia malayi. *Parasites and Vectors,* 5.

LANGROVÁ, I., MAKOVCOVÁ, K., VADLEJCH, J., JANKOVSKÁ, I., PETRTÝL, M., FECHTNER, J., KEIL, P., LYTVYNETS, A. & BORKOVCOVÁ, M. 2008. Arrested development of sheep strongyles: onset and resumption under field conditions of Central Europe. *Parasitology Research,* 103**,** 387-392.

LEATHWICK, D. M., VLASSOFF, A. & BARLOW, N. D. 1995. A model for nematodiasis in New Zealand lambs: the effect of drenching regime and grazing management on the development of anthelmintic resistance. *Int J Parasitol,* 25**,** 1479-90.

LEE, R. C., FEINBAUM, R. L. & AMBROS, V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell,* 75**,** 843-54.

LEE, R. C., HAMMELL, C. M. & AMBROS, V. 2006. Interacting endogenous and exogenous RNAi pathways in Caenorhabditis elegans. *RNA (New York, N.Y.),* 12**,** 589-97.

LIEGEOIS, S., BENEDETTO, A., GARNIER, J. M., SCHWAB, Y. & LABOUESSE, M. 2006. The V0-ATPase mediates apical secretion of exosomes containing Hedgehog-related proteins in Caenorhabditis elegans. *J Cell Biol,* 173**,** 949-61.

LITTLE, P. R., HODGES, A., WATSON, T. G., SEED, J. A. & MAEDER, S. J. 2010. Field efficacy and safety of an oral formulation of the novel combination anthelmintic, derquantel-abamectin, in sheep in New Zealand. *N Z Vet J,* 58**,** 121-9.

LU, G., VILLALBA, M., COSCIA, M., HOFFMAN, D. & KING, T. 1993. Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen

5, from hornets, wasps, and yellow jackets. *The Journal of Immunology,* 150**,** 2823-2830.
MAEDA, I., KOHARA, Y., YAMAMOTO, M. & SUGIMOTO, A. 2001. Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. *Current biology : CB,* 11**,** 171-176.
MANIAR, J. M. & FIRE, A. Z. 2011. EGO-1, a *C. elegans* RdRP, Modulates Gene Expression via Production of mRNA-Templated Short Antisense RNAs. *Current Biology,* 21**,** 449-459.
MANSOUR, M. M., DIXON, J. B., ROWAN, T. G. & CARTER, S. D. 1992. Modulation of calf immune responses by Ostertagia ostertagi: the effect of diet during trickle infection. *Vet Immunol Immunopathol,* 33**,** 261-9.
MARCILLA, A., TRELIS, M., CORTES, A., SOTILLO, J., CANTALAPIEDRA, F., MINGUEZ, M. T., VALERO, M. L., SANCHEZ DEL PINO, M. M., MUNOZ-ANTOLI, C., TOLEDO, R. & BERNAL, D. 2012. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. *PLoS ONE,* 7**,** e45974.
MARTIN, R. J., VALKANOV, M. A., DALE, V. M., ROBERTSON, A. P. & MURRAY, I. 1996. Electrophysiology of Ascaris muscle and anti-nematodal drug action. *Parasitology,* 113 Suppl**,** S137-56.
MARTIN, R. J. 1997. Modes of action of anthelmintic drugs. *Vet J,* 154**,** 11-34.
MATHIVANAN, S. & SIMPSON, R. J. 2009. ExoCarta: A compendium of exosomal proteins and RNA. *Proteomics,* 9**,** 4997-5000.
MATHIVANAN, S., JI, H. & SIMPSON, R. J. 2010. Exosomes: Extracellular organelles important in intercellular communication. *Journal of Proteomics,* 73**,** 1907-1920.
MATHIVANAN, S., FAHNER, C. J., REID, G. E. & SIMPSON, R. J. 2012. ExoCarta 2012: database of exosomal proteins, RNA and lipids. *Nucleic Acids Res,* 40**,** D1241-4.
MATTHEWS, J. B., DAVIDSON, A. J., FREEMAN, K. L. & FRENCH, N. P. 2001. Immunisation of cattle with recombinant acetylcholinesterase from Dictyocaulus viviparus and with adult worm ES products. *Int J Parasitol,* 31**,** 307-17.
MATZKE, M., MATZKE, A. J. M. & KOOTER, J. M. 2001. RNA: Guiding Gene Silencing. *Science,* 293**,** 1080-1083.
MAULE, A. G., MCVEIGH, P., DALZELL, J. J., ATKINSON, L., MOUSLEY, A. & MARKS, N. J. 2011. An eye on RNAi in nematode parasites. *Trends in parasitology,* 27**,** 505-513.
MCKAY, D. M. & BIENENSTOCK, J. 1994. The interaction between mast cells and nerves in the gastrointestinal tract. *Immunol Today,* 15**,** 533-8.
MCKEAND, J. B. 2000. Vaccine development and diagnostics of Dictyocaulus viviparus. *Parasitology,* 120 Suppl**,** S17-23.
MCSORLEY, H. J., GRAINGER, J. R., HARCUS, Y., MURRAY, J., NISBET, A. J., KNOX, D. P. & MAIZELS, R. M. 2010. daf-7-related TGF-beta homologues from Trichostrongyloid nematodes show contrasting life-cycle expression patterns. *Parasitology,* 137**,** 159-71.
MEDZHITOV, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature,* 449**,** 819-26.

MENDES, R. E., PEREZ-ECIJA, R. A., ZAFRA, R., BUFFONI, L., MARTINEZ-MORENO, A., DALTON, J. P., MULCAHY, G. & PEREZ, J. 2010. Evaluation of hepatic changes and local and systemic immune responses in goats immunized with recombinant Peroxiredoxin (Prx) and challenged with Fasciola hepatica. *Vaccine,* 28**,** 2832-40.

MENON, R., GASSER, R. B., MITREVA, M. & RANGANATHAN, S. 2012. An analysis of the transcriptome of *Teladorsagia circumcincta*: its biological and biotechnological implications. *BMC Genomics,* 13**,** 17pp.-17pp.

MEYVIS, Y., GELDHOF, P., GEVAERT, K., TIMMERMAN, E., VERCRUYSSE, J. & CLAEREBOUT, E. 2007. Vaccination against Ostertagia ostertagi with subfractions of the protective ES-thiol fraction. *Vet Parasitol,* 149**,** 239-45.

MICHEL, J. F. 1978. Topical themes in the study of arrested development. *In:* BORGSTEEDE, F. H. (ed.) *Facts and Reflections. III. Workshop on Arrested Development of Nematodes of Sheep and Cattle.* Lelystad, Netherlands: Central Veterinary Institute.

MILLER, H. R. 1996. Prospects for the immunological control of ruminant gastrointestinal nematodes: natural immunity, can it be harnessed? *Int J Parasitol,* 26**,** 801-11.

MILLER, T. A. 1964. Effect of X-irradiation upon the infective larvae of Ancylostoma caninum and the immunogenic effect in dogs of a single infection with 40 kr-irradiated larvae. *J Parasitol,* 50**,** 735-42.

MILLER, T. A. 1971. Vaccination against the canine hookworm diseases. *Adv Parasitol,* 9**,** 153-83.

MILLER, T. A. 1978. Industrial development and field use of the canine hookworm vaccine. *Adv Parasitol,* 16**,** 333-42.

MONTANER, S., GALIANO, A., TRELIS, M., MARTIN-JAULAR, L., DEL PORTILLO, H. A., BERNAL, D. & MARCILLA, A. 2014. The Role of Extracellular Vesicles in Modulating the Host Immune Response during Parasitic Infections. *Frontiers in immunology,* 5**,** 433.

MURPHY, E. V., ZHANG, Y., ZHU, W. & BIGGS, J. 1995. The human glioma pathogenesis-related protein is structurally related to plant pathogenesis-related proteins and its gene is expressed specifically in brain tumors. *Gene,* 159**,** 131-5.

NAPOLI, C., LEMIEUX, C. & JORGENSEN, R. 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *The Plant Cell Online,* 2**,** 279-289.

NEWTON, S. E., MORRISH, L. E., MARTIN, P. J., MONTAGUE, P. E. & ROLPH, T. P. 1995. Protection against multiply drug-resistant and geographically distant strains of Haemonchus contortus by vaccination with H11, a gut membrane-derived protective antigen. *Int J Parasitol,* 25**,** 511-21.

NIEUWHOF, G. J. & BISHOP, S. C. 2005. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. *Animal Science,* 81**,** 23-29.

NIEZEN, J. H., WAGHORN, G. C. & CHARLESTON, W. A. 1998. Establishment and fecundity of Ostertagia circumcincta and Trichostrongylus colubriformis in lambs fed lotus (Lotus pedunculatus) or perennial ryegrass. *Vet Parasitol,* 78**,** 13-21.

NISBET, A. J., REDMOND, D. L., MATTHEWS, J. B., WATKINS, C., YAGA, R., JONES, J. T., NATH, M. & KNOX, D. P. 2008. Stage-specific gene expression in *Teladorsagia circumcincta* (Nematoda: Strongylida) infective larvae and early parasitic stages. *International Journal for Parasitology,* 38**,** 829-838.

NISBET, A. J., KNOX, D. P., MCNAIR, C. M., MEIKLE, L. I., SMITH, S. K., WILDBLOOD, L. A. & MATTHEWS, J. B. 2009. Immune recognition of the surface associated antigen, Tc-SAA-1, from infective larvae of *Teladorsagia circumcincta*. *Parasite Immunology,* 31**,** 32-40.

NISBET, A. J., BELL, N. E. V., MCNEILLY, T. N., KNOX, D. P., MAIZELS, R. M., MEIKLE, L. I., WILDBLOOD, L. A. & MATTHEWS, J. B. 2010a. A macrophage migration inhibitory factor-like tautomerase from *Teladorsagia circumcincta* (Nematoda: Strongylida). *Parasite Immunology,* 32**,** 503-511.

NISBET, A. J., SMITH, S. K., ARMSTRONG, S., MEIKLE, L. I., WILDBLOOD, L. A., BEYNON, R. J. & MATTHEWS, J. B. 2010b. *Teladorsagia circumcincta*: Activation-associated secreted proteins in excretory/secretory products of fourth stage larvae are targets of early IgA responses in infected sheep. *Experimental Parasitology,* 125**,** 329-337.

NISBET, A. J., ZARLENGA, D. S., KNOX, D. P., MEIKLE, L. I., WILDBLOOD, L. A. & MATTHEWS, J. B. 2011. A calcium-activated apyrase from *Teladorsagia circumcincta*: an excretory/secretory antigen capable of modulating host immune responses? *Parasite Immunology,* 33**,** 236-243.

NISBET, A. J., MCNEILLY, T. N., WILDBLOOD, L. A., MORRISON, A. A., BARTLEY, D. J., BARTLEY, Y., LONGHI, C., MCKENDRICK, I. J., PALAREA-ALBALADEJO, J. & MATTHEWS, J. B. 2013. Successful immunization against a parasitic nematode by vaccination with recombinant proteins. *Vaccine,* 31**,** 4017-4023.

O'NEILL, S. M., MILLS, K. H. & DALTON, J. P. 2001. Fasciola hepatica cathepsin L cysteine proteinase suppresses Bordetella pertussis-specific interferon-gamma production in vivo. *Parasite immunology,* 23**,** 541-7.

OSTROWSKI, M., CARMO, N. B., KRUMEICH, S., FANGET, I., RAPOSO, G., SAVINA, A., MOITA, C. F., SCHAUER, K., HUME, A. N., FREITAS, R. P., GOUD, B., BENAROCH, P., HACOHEN, N., FUKUDA, M., DESNOS, C., SEABRA, M. C., DARCHEN, F., AMIGORENA, S., MOITA, L. F. & THERY, C. 2010. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol,* 12**,** 19-30; sup pp 1-13.

PAK, J. & FIRE, A. 2007. Distinct Populations of Primary and Secondary Effectors During RNAi in *C. elegans*. *Science,* 315**,** 241-244.

PAN, B. T. & JOHNSTONE, R. M. 1983. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell,* 33**,** 967-78.

PARAUD, C. & CHARTIER, C. 2003. Biological control of infective larvae of a gastro-intestinal nematode (Teladorsagia circumcincta) and a small lungworm (Muellerius capillaris) by Duddingtonia flagrans in goat faeces. *Parasitol Res,* 89**,** 102-6.

PARKINSON, J., MITREVA, M., WHITTON, C., THOMSON, M., DAUB, J., MARTIN, J., SCHMID, R., HALL, N., BARRELL, B., WATERSTON, R.

H., MCCARTER, J. P. & BLAXTER, M. L. 2004. A transcriptomic analysis of the phylum Nematoda. *Nature Genetics,* 36**,** 1259-1267.

PASQUINELLI, A. E., REINHART, B. J., SLACK, F., MARTINDALE, M. Q., KURODA, M. I., MALLER, B., HAYWARD, D. C., BALL, E. E., DEGNAN, B., MULLER, P., SPRING, J., SRINIVASAN, A., FISHMAN, M., FINNERTY, J., CORBO, J., LEVINE, M., LEAHY, P., DAVIDSON, E. & RUVKUN, G. 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature,* 408**,** 86-9.

POPE, S. M. & LASSER, C. 2013. Toxoplasma gondii infection of fibroblasts causes the production of exosome-like vesicles containing a unique array of mRNA and miRNA transcripts compared to serum starvation. *Journal of extracellular vesicles,* 2.

PRICHARD, R. 1994. Anthelmintic resistance. *Vet Parasitol,* 54**,** 259-68.

PRICHARD, R. K. 1990. Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. *Int J Parasitol,* 20**,** 515-23.

RAEYMAEKERS, L. 1995. A commentary on the practical applications of competitive PCR. *Genome Research,* 5**,** 91-94.

RAINA, O. K., NAGAR, G., VARGHESE, A., PRAJITHA, G., ALEX, A., MAHARANA, B. R. & JOSHI, P. 2011. Lack of protective efficacy in buffaloes vaccinated with Fasciola gigantica leucine aminopeptidase and peroxiredoxin recombinant proteins. *Acta tropica,* 118**,** 217-22.

RAPOSO, G., NIJMAN, H. W., STOORVOGEL, W., LIEJENDEKKER, R., HARDING, C. V., MELIEF, C. J. & GEUZE, H. J. 1996. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med,* 183**,** 1161-72.

REDMOND, D. L., SMITH, S. K., HALLIDAY, A., SMITH, W. D., JACKSON, F., KNOX, D. P. & MATTHEWS, J. B. 2006. An immunogenic cathepsin F secreted by the parasitic stages of *Teladorsagia circumcincta*. *International Journal for Parasitology,* 36**,** 277-286.

REHMAN, A. & JASMER, D. P. 1998. A tissue specific approach for analysis of membrane and secreted protein antigens from Haemonchus contortus gut and its application to diverse nematode species. *Mol Biochem Parasitol,* 97**,** 55-68.

REINHART, B. J., SLACK, F. J., BASSON, M., PASQUINELLI, A. E., BETTINGER, J. C., ROUGVIE, A. E., HORVITZ, H. R. & RUVKUN, G. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature,* 403**,** 901-6.

ROBERTSON, A. P., CLARK, C. L., BURNS, T. A., THOMPSON, D. P., GEARY, T. G., TRAILOVIC, S. M. & MARTIN, R. J. 2002. Paraherquamide and 2-deoxy-paraherquamide distinguish cholinergic receptor subtypes in Ascaris muscle. *J Pharmacol Exp Ther,* 302**,** 853-60.

ROBINSON, M. W., HUTCHINSON, A. T., DALTON, J. P. & DONNELLY, S. 2010a. Peroxiredoxin: a central player in immune modulation. *Parasite immunology,* 32**,** 305-13.

ROBINSON, M. W., HUTCHINSON, A. T., DONNELLY, S. & DALTON, J. P. 2010b. Worm secretory molecules are causing alarm. *Trends Parasitol,* 26**,** 371-2.

ROMANO, N. & MACINO, G. 1992. Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Molecular Microbiology,* 6**,** 3343-3353.

SACKETT, D. L. & VARMA, J. K. 1993. Molecular mechanism of colchicine action: induced local unfolding of beta-tubulin. *Biochemistry,* 32**,** 13560-5.

SAKKAS, P., HOUDIJK, J. G., ATHANASIADOU, S. & KYRIAZAKIS, I. 2012. Sensitivity of periparturient breakdown of immunity to parasites to dietary protein source. *J Anim Sci,* 90**,** 3954-62.

SAMARASINGHE, B., KNOX, D. P. & BRITTON, C. 2011. Factors affecting susceptibility to RNA interference in *Haemonchus contortus* and in vivo silencing of an H11 aminopeptidase gene. *International Journal for Parasitology,* 41**,** 51-59.

SANGSTER, N. C., RILEY, F. L. & WILEY, L. J. 1998. Binding of [3H]m-aminolevamisole to receptors in levamisole-susceptible and -resistant Haemonchus contortus. *Int J Parasitol,* 28**,** 707-17.

SARGISON, N. D., JACKSON, F., BARTLEY, D. J., WILSON, D. J., STENHOUSE, L. J. & PENNY, C. D. 2007. Observations on the emergence of multiple anthelmintic resistance in sheep flocks in the south-east of Scotland. *Veterinary Parasitology,* 145**,** 65-76.

SARGISON, N. D. 2011. Pharmaceutical Control of Endoparasitic Helminth Infections in Sheep. *The Veterinary clinics of North America. Food animal practice,* 27**,** 139-156.

SAVIN, K. W., DOPHEIDE, T. A., FRENKEL, M. J., WAGLAND, B. M., GRANT, W. N. & WARD, C. W. 1990. Characterization, cloning and host-protective activity of a 30-kilodalton glycoprotein secreted by the parasitic stages of Trichostrongylus colubriformis. *Mol Biochem Parasitol,* 41**,** 167-76.

SAVINA, A., FADER, C. M., DAMIANI, M. T. & COLOMBO, M. I. 2005. Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. *Traffic,* 6**,** 131-43.

SCHALLIG, H. D., VAN LEEUWEN, M. A. & HENDRIKX, W. M. 1995. Isotype-specific serum antibody responses of sheep to Haemonchus contortus antigens. *Vet Parasitol,* 56**,** 149-62.

SCHALLIG, H. D., VAN LEEUWEN, M. A. & CORNELISSEN, A. W. 1997a. Protective immunity induced by vaccination with two Haemonchus contortus excretory secretory proteins in sheep. *Parasite Immunol,* 19**,** 447-53.

SCHALLIG, H. D. F. H. & VAN LEEUWEN, M. A. W. 1997. Protective immunity to the blood-feeding nematode *Haemonchus contortus* induced by vaccination with parasite low molecular weight antigens. *Parasitology,* 114**,** 293-299.

SCHALLIG, H. D. F. H., VAN LEEUWEN, M. A. W., VERSTREPEN, B. E. & CORNELISSEN, A. W. C. A. 1997b. Molecular characterization and expression of two putative protective excretory secretory proteins of *Haemonchus contortus*. *Molecular and Biochemical Parasitology,* 88**,** 203-213.

SCHAPPI, J. M., KRBANJEVIC, A. & RASENICK, M. M. 2014. Tubulin, actin and heterotrimeric G proteins: coordination of signaling and structure. *Biochimica et biophysica acta,* 1838**,** 674-81.

SCHMID-HEMPEL, P. 2008. Parasite immune evasion: a momentous molecular war. *Trends Ecol Evol,* 23**,** 318-26.
SCOTT, I., POMROY, W. E., KENYON, P. R., SMITH, G., ADLINGTON, B. & MOSS, A. 2013. Lack of efficacy of monepantel against Teladorsagia circumcincta and Trichostrongylus colubriformis. *Vet Parasitol,* 198**,** 166-71.
SCOTT, P. R. 2007. *Sheep Medicine,* Manson Publishing Ltd.
SEATON, D. S., JACKSON, F., SMITH, W. D. & ANGUS, K. W. 1989. Development of immunity to incoming radiolabelled larvae in lambs continuously infected with *Ostertagia circumcincta*. *Research in Veterinary Science,* 46**,** 241-246.
SEN, L., GHOSH, K., BIN, Z., QIANG, S., THOMPSON, M. G., HAWDON, J. M., KOSKI, R. A., SHUHUA, X. & HOTEZ, P. J. 2000. Hookworm burden reductions in BALB/c mice vaccinated with recombinant Ancylostoma secreted proteins (ASPs) from Ancylostoma duodenale, Ancylostoma caninum and Necator americanus. *Vaccine,* 18**,** 1096-102.
SHEVCHENKO, A., WILM, M., VORM, O. & MANN, M. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem,* 68**,** 850-8.
SHOMPOLE, S., YAO, C., CHENG, X., KNOX, D., JOHNSON, S. & JASMER, D. P. 2002. Distinct characteristics of two intestinal protein compartments discriminated by using fenbendazole and a benzimidazole resistant isolate of Haemonchus contortus. *Exp Parasitol,* 101**,** 200-9.
SIJEN, T., FLEENOR, J., SIMMER, F., THIJSSEN, K. L., PARRISH, S., TIMMONS, L., PLASTERK, R. H. A. & FIRE, A. 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell,* 107**,** 465-476.
SIJEN, T., STEINER, F. A., THIJSSEN, K. L. & PLASTERK, R. H. A. 2007. Secondary siRNAs Result from Unprimed RNA Synthesis and Form a Distinct Class. *Science,* 315**,** 244-247.
SILVERMAN, J. M., CLOS, J., HORAKOVA, E., WANG, A. Y., WIESGIGL, M., KELLY, I., LYNN, M. A., MCMASTER, W. R., FOSTER, L. J., LEVINGS, M. K. & REINER, N. E. 2010. Leishmania exosomes modulate innate and adaptive immune responses through effects on monocytes and dendritic cells. *J Immunol,* 185**,** 5011-22.
SIMONS, M. & RAPOSO, G. 2009. Exosomes – vesicular carriers for intercellular communication. *Current Opinion in Cell Biology,* 21**,** 575-581.
SIMPSON, H. V. 2000. Pathophysiology of Abomasal Parasitism: Is the Host or Parasite Responsible? *The Veterinary Journal,* 160**,** 177-191.
SIMPSON, R. J., LIM, J. W., MORITZ, R. L. & MATHIVANAN, S. 2009. Exosomes: proteomic insights and diagnostic potential. *Expert Rev Proteomics,* 6**,** 267-83.
SIMPSON, R. J., KALRA, H. & MATHIVANAN, S. 2012. ExoCarta as a resource for exosomal research. *J Extracell Vesicles,* 1.
SINGLETON, D. R., STEAR, M. J. & MATTHEWS, L. 2011. A mechanistic model of developing immunity to Teladorsagia circumcincta infection in lambs. *Parasitology,* 138**,** 322-32.
SMARDON, A., SPOERKE, J. M., STACEY, S. C., KLEIN, M. E., MACKIN, N. & MAINE, E. M. 2000. EGO-1 is related to RNA-directed RNA polymerase

and functions in germ-line development and RNA interference in *C. elegans*. *Current Biology,* 10**,** 169-178.

SMITH, A. M., DOWD, A. J., HEFFERNAN, M., ROBERTSON, C. D. & DALTON, J. P. 1993a. Fasciola hepatica: a secreted cathepsin L-like proteinase cleaves host immunoglobulin. *International journal for parasitology,* 23**,** 977-83.

SMITH, A. M., DOWD, A. J., MCGONIGLE, S., KEEGAN, P. S., BRENNAN, G., TRUDGETT, A. & DALTON, J. P. 1993b. Purification of a cathepsin L-like proteinase secreted by adult Fasciola hepatica. *Molecular and biochemical parasitology,* 62**,** 1-8.

SMITH, S. K. & SMITH, W. D. 1996. Immunisation of sheep with an integral membrane glycoprotein complex of Haemonchus contortus and with its major polypeptide components. *Res Vet Sci,* 60**,** 1-6.

SMITH, S. K., PETTIT, D., NEWLANDS, G. F., REDMOND, D. L., SKUCE, P. J., KNOX, D. P. & SMITH, W. D. 1999. Further immunization and biochemical studies with a protective antigen complex from the microvillar membrane of the intestine of Haemonchus contortus. *Parasite Immunol,* 21**,** 187-99.

SMITH, S. K., NISBET, A. J., MEIKLE, L. I., INGLIS, N. F., SALES, J., BEYNON, R. J. & MATTHEWS, J. B. 2009. Proteomic analysis of excretory/secretory products released by *Teladorsagia circumcincta* larvae early post-infection. *Parasite Immunology,* 31**,** 10-19.

SMITH, T. S., MUNN, E. A., GRAHAM, M., TAVERNOR, A. S. & GREENWOOD, C. A. 1993c. Purification and evaluation of the integral membrane protein H11 as a protective antigen against Haemonchus contortus. *Int J Parasitol,* 23**,** 271-80.

SMITH, T. S., GRAHAM, M., MUNN, E. A., NEWTON, S. E., KNOX, D. P., COADWELL, W. J., MCMICHAEL-PHILLIPS, D., SMITH, H., SMITH, W. D. & OLIVER, J. J. 1997. Cloning and characterization of a microsomal aminopeptidase from the intestine of the nematode Haemonchus contortus. *Biochim Biophys Acta,* 1338**,** 295-306.

SMITH, W. D., JACKSON, E. & JACKSON, F. 1982. Attempts to immunise sheep against Ostertagia circumcincta with irradiated larvae. *Res Vet Sci,* 32**,** 101-5.

SMITH, W. D., JACKSON, F., JACKSON, E. & WILLIAMS, J. 1983a. Local immunity and *Ostertagia circumcincta*: Changes in the gastric lymph of sheep after a primary infection. *Journal of Comparative Pathology,* 93**,** 471-478.

SMITH, W. D., JACKSON, F., JACKSON, E. & WILLIAMS, J. 1983b. Local immunity and *Ostertagia circumcingta*: Changes in the gastric lymph of immune sheep after a challenge infection. *Journal of Comparative Pathology,* 93**,** 479-488.

SMITH, W. D., JACKSON, F., JACKSON, E., WILLIAMS, J. & MILLER, H. R. P. 1984. Manifestations of resistance to ovine ostertagiasis associated with immunological responses in the gastric lymph. *Journal of Comparative Pathology,* 94**,** 591-601.

SMITH, W. D., JACKSON, F., JACKSON, E. & WILLIAMS, J. 1985. Age immunity to *Ostertagia circumcincta*: Comparison of the local immune

responses of 4 1/2- and 10-month-old lambs. *Journal of Comparative Pathology,* 95**,** 235-245.

SMITH, W. D., JACKSON, F., JACKSON, E., GRAHAM, R., WILLIAMS, J., WILLADSEN, S. M. & FEHILLY, C. B. 1986. Transfer of immunity to *Ostertagia circumcincta* and IgA memory between identical sheep by lymphocytes collected from gastric lymph. *Research in Veterinary Science,* 41**,** 300-306.

SMITH, W. D. 1988. Mechanisms of Immunity to Gastrointestinal Nematodes of Sheep. *In:* THOMSON, E. F. & THOMSON, F. S. (eds.) *Increasing Small Ruminant Productivity in Semi-arid Areas.* Springer Netherlands.

SMITH, W. D. 1993. Protection in lambs immunised with Haemonchus contortus gut membrane proteins. *Res Vet Sci,* 54**,** 94-101.

SMITH, W. D. & SMITH, S. K. 1993. Evaluation of aspects of the protection afforded to sheep immunised with a gut membrane protein of Haemonchus contortus. *Res Vet Sci,* 55**,** 1-9.

SMITH, W. D., SMITH, S. K. & MURRAY, J. M. 1994. Protection studies with integral membrane fractions of Haemonchus contortus. *Parasite Immunol,* 16**,** 231-41.

SMITH, W. D. 1999. Prospects for vaccines of helminth parasites of grazing ruminants. *Int J Parasitol,* 29**,** 17-24.

SMITH, W. D., PETTIT, D. & SMITH, S. K. 2001. Cross-protection studies with gut membrane glycoprotein antigens from Haemonchus contortus and Teladorsagia circumcincta. *Parasite Immunol,* 23**,** 203-11.

SMITH, W. D. 2007. Some observations on immunologically mediated inhibited Teladorsagia circumcincta and their subsequent resumption of development in sheep. *Veterinary parasitology,* 147**,** 103-9.

SOMMERVILLE, R. I. 1953. Development of Ostertagia circumcincta in the abomasal mucosa of the sheep. *Nature,* 171**,** 482-3.

SONG, J.-J., SMITH, S. K., HANNON, G. J. & JOSHUA-TOR, L. 2004. Crystal Structure of Argonaute and Its Implications for RISC Slicer Activity. *Science,* 305**,** 1434-1437.

STEAR, M. J., BISHOP, S. C., DOLIGALSKA, M., DUNCAN, J. L., HOLMES, P. H., IRVINE, J., MCCRIRIE, L., MCKELLAR, Q. A., SINSKI, E. & MURRAY, M. A. X. 1995. Regulation of egg production, worm burden, worm length and worm fecundity by host responses in sheep infected with *Ostertagia circumcincta*. *Parasite Immunology,* 17**,** 643-652.

STEAR, M. J., STRAIN, S. & BISHOP, S. C. 1999a. How lambs control infection with *Ostertagia circumcincta*. *Veterinary Immunology and Immunopathology,* 72**,** 213-218.

STEAR, M. J., STRAIN, S. & BISHOP, S. C. 1999b. Mechanisms underlying resistance to nematode infection. *International Journal for Parasitology,* 29**,** 51-56.

STEAR, M. J., FITTON, L., INNOCENT, G. T., MURPHY, L., RENNIE, K. & MATTHEWS, L. 2007. The dynamic influence of genetic variation on the susceptibility of sheep to gastrointestinal nematode infection. *J R Soc Interface,* 4**,** 767-76.

STENMARK, H. 2009. Rab GTPases as coordinators of vesicle traffic. *Nature reviews. Molecular cell biology,* 10**,** 513-25.

STEVENSON, L. M., HUNTLEY, J. F., SMITH, W. D. & JONES, D. G. 1994. Local eosinophil- and mast cell-related responses in abomasal nematode infections of lambs. *FEMS Immunol Med Microbiol,* 8**,** 167-73.

STRAIN, S. A., BISHOP, S. C., HENDERSON, N. G., KERR, A., MCKELLAR, Q. A., MITCHELL, S. & STEAR, M. J. 2002. The genetic control of IgA activity against Teladorsagia circumcincta and its association with parasite resistance in naturally infected sheep. *Parasitology,* 124**,** 545-52.

SUTHERLAND, I. A., BROWN, A. E., GREEN, R. S., MILLER, C. M. & LEATHWICK, D. M. 1999. The immune response of sheep to larval challenge with Ostertagia circumcincta and O. ostertagi. *Vet Parasitol,* 84**,** 125-35.

TABARA, H., GRISHOK, A. & MELLO, C. C. 1998. RNAi in *C. elegans* - Soaking in the genome sequence. *Science,* 282**,** 430-431.

TABARA, H., SARKISSIAN, M., KELLY, W. G., FLEENOR, J., GRISHOK, A., TIMMONS, L., FIRE, A. & MELLO, C. C. 1999. The rde-1 Gene, RNA Interference, and Transposon Silencing in *C. elegans*. *Cell,* 99**,** 123-132.

TABARA, H., YIGIT, E., SIOMI, H. & MELLO, C. C. 2002. The dsRNA Binding Protein RDE-4 Interacts with RDE-1, DCR-1, and a DExH-Box Helicase to Direct RNAi in *C. elegans*. *Cell,* 109**,** 861-871.

TAKATS, C., SCHALLIG, H. D., VAN LEEUWEN, M. A. & HENDRIKX, W. M. 1995. Immune responses of sheep to microdissected parts of Haemonchus contortus. *Int J Parasitol,* 25**,** 857-60.

TARRAB-HAZDAI, R., CAMACHO, M., MENDELOVIC, F. & SCHECHTMAN, D. 1997. An association between activity of the Na/K-pump and resistance of Schistosoma mansoni towards complement-mediated killing. *Parasite immunology,* 19**,** 395-400.

TAWE, W., PEARLMAN, E., UNNASCH, T. R. & LUSTIGMAN, S. 2000. Angiogenic activity of *Onchocerca volvulus* recombinant proteins similar to vespid venom antigen 5. *Molecular and Biochemical Parasitology,* 109**,** 91-99.

TAYLOR, G. K. & GOODLETT, D. R. 2005. Rules governing protein identification by mass spectrometry. *Rapid Commun Mass Spectrom,* 19**,** 3420.

TAYLOR, M. A., COOP, R. L. & WALL, R. L. 2007. *Veterinary Parasitology*, Blackwell Publishing.

THERY, C., REGNAULT, A., GARIN, J., WOLFERS, J., ZITVOGEL, L., RICCIARDI-CASTAGNOLI, P., RAPOSO, G. & AMIGORENA, S. 1999. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J Cell Biol,* 147**,** 599-610.

THERY, C., BOUSSAC, M., VERON, P., RICCIARDI-CASTAGNOLI, P., RAPOSO, G., GARIN, J. & AMIGORENA, S. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol,* 166**,** 7309-18.

THERY, C., DUBAN, L., SEGURA, E., VERON, P., LANTZ, O. & AMIGORENA, S. 2002. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol,* 3**,** 1156-62.

THERY, C., AMIGORENA, S., RAPOSO, G. & CLAYTON, A. 2006. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol,* Chapter 3**,** Unit 3 22.

THERY, C. 2011. Exosomes: secreted vesicles and intercellular communications. *F1000 Biol Rep,* 3**,** 15.

THREADGOLD, L. T. 1963. The tegument and associated structures of Fasciola hepatica. *Quarterly Journal of Microscopical Science,* s3-104**,** 505-512.

THRELKELD, W. L. 1934. The life history of Ostertagia circumcincta. Virginia Polytechnic Institute, Virginia Agricultural Experimental Station: Blacksburg, Va.

TIJSTERMAN, M., MAY, R. C., SIMMER, F., OKIHARA, K. L. & PLASTERK, R. H. A. 2004. Genes required for systemic RNA interference in *Caenorhabditis elegans*. *Current Biology,* 14**,** 111-116.

TIMMONS, L. & FIRE, A. 1998. Specific interference by ingested dsRNA. *Nature,* 395**,** 854-854.

TZELOS, T., MATTHEWS, J. B., WHITELAW, B. & KNOX, D. P. 2013. Marker genes for activation of the RNA interference (RNAi) pathway in the free-living nematode Caenorhabditis elegans and RNAi development in the ovine nematode Teladorsagia circumcincta. *J Helminthol***,** 1-9.

VALADI, H., EKSTROM, K., BOSSIOS, A., SJOSTRAND, M., LEE, J. J. & LOTVALL, J. O. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol,* 9**,** 654-9.

VALDERRABANO, J., DELFA, R. & URIARTE, J. 2002. Effect of level of feed intake on the development of gastrointestinal parasitism in growing lambs. *Vet Parasitol,* 104**,** 327-38.

VAN BLOKLAND, R., VAN DER GEEST, N., MOL, J. N. M. & KOOTER, J. M. 1994. Transgene-mediated suppression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. *The Plant Journal,* 6**,** 861-877.

VAN LOON, L. C., REP, M. & PIETERSE, C. M. 2006. Significance of inducible defense-related proteins in infected plants. *Annu Rev Phytopathol,* 44**,** 135-62.

VAN NIEL, G., PORTO-CARREIRO, I., SIMOES, S. & RAPOSO, G. 2006. Exosomes: a common pathway for a specialized function. *J Biochem,* 140**,** 13-21.

VAN WYK, J. A. 2001. Refugia--overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. *Onderstepoort J Vet Res,* 68**,** 55-67.

VANCE, V. & VAUCHERET, H. 2001. RNA Silencing in Plants--Defense and Counterdefense. *Science,* 292**,** 2277-2280.

VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, A. & SPELEMAN, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology,* 3**,** research0034.1 - research0034.11.

VERVELDE, L., VAN LEEUWEN, M. A., KRUIDENIER, M., KOOYMAN, F. N., HUNTLEY, J. F., VAN DIE, I. & CORNELISSEN, A. W. 2002. Protection

studies with recombinant excretory/secretory proteins of Haemonchus contortus. *Parasite Immunol,* 24**,** 189-201.
VINEY, M. E. & THOMPSON, F. J. 2008. Two hypotheses to explain why RNA interference does not work in animal parasitic nematodes. *International Journal for Parasitology,* 38**,** 43-47.
VISSER, A., GELDHOF, P., DE MAERE, V., KNOX, D. P., VERCRUYSSE, J. & CLAEREBOUT, E. 2006. Efficacy and specificity of RNA interference in larval life-stages of *Ostertagia ostertagi*. *Parasitology,* 133**,** 777-783.
VISSER, A. 2008. *Characterisation of activation-associated secreted proteins (ASP) in the bovine abomasal nematode Ostertagia ostertagi.* PhD Thesis, Ghent University.
VISSER, A., VAN ZEVEREN, A. M., MEYVIS, Y., PEELAERS, I., VAN DEN BROECK, W., GEVAERT, K., VERCRUYSSE, J., CLAEREBOUT, E. & GELDHOF, P. 2008. Gender-enriched transcription of activation associated secreted proteins in *Ostertagia ostertagi*. *International Journal for Parasitology,* 38**,** 455-465.
VON SAMSON-HIMMELSTJERNA, G., BLACKHALL, W. J., MCCARTHY, J. S. & SKUCE, P. J. 2007. Single nucleotide polymorphism (SNP) markers for benzimidazole resistance in veterinary nematodes. *Parasitology,* 134**,** 1077-86.
WALLER, P. J. & LARSEN, M. 1993. The role of nematophagous fungi in the biological control of nematode parasites of livestock. *Int J Parasitol,* 23**,** 539-46.
WANG, J. & KIM, S. K. 2003. Global analysis of dauer gene expression in *Caenorhabditis elegans*. *Development,* 130**,** 1621-1634.
WANG, J., WU, M., WANG, B. & HAN, Z. 2013. Comparison of the RNA interference effects triggered by dsRNA and siRNA in Tribolium castaneum. *Pest management science,* 69**,** 781-6.
WINDON, R. G., DINEEN, J. K., GREGG, P., GRIFFITHS, D. A. & DONALD, A. D. 1984. The role of thresholds in the response of lambs to vaccination with irradiated Trichostrongylus colubriformis larvae. *Int J Parasitol,* 14**,** 423-8.
WINDON, R. G. 1996. Genetic control of resistance to helminths in sheep. *Vet Immunol Immunopathol,* 54**,** 245-54.
WINSTON, W. M., MOLODOWITCH, C. & HUNTER, C. P. 2002. Systemic RNAi in *C. elegans* Requires the Putative Transmembrane Protein SID-1. *Science,* 295**,** 2456-2459.
WINSTON, W. M., SUTHERLIN, M., WRIGHT, A. J., FEINBERG, E. H. & HUNTER, C. P. 2007. *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. *Proceedings of the National Academy of Sciences,* 104**,** 10565-10570.
WOLSTENHOLME, A. J., FAIRWEATHER, I., PRICHARD, R., VON SAMSON-HIMMELSTJERNA, G. & SANGSTER, N. C. 2004. Drug resistance in veterinary helminths. *Trends Parasitol,* 20**,** 469-76.
WOOLASTON, R. R. & BAKER, R. L. 1996. Prospects of breeding small ruminants for resistance to internal parasites. *Int J Parasitol,* 26**,** 845-55.
WRIGLEY, J., MCARTHUR, M., MCKENNA, P. B. & MARIADASS, B. 2006. Resistance to a triple combination of broad-spectrum anthelmintics in

naturally-acquired *Ostertagia circumcincta* infections in sheep. *New Zealand Veterinary Journal,* 54**,** 47-49.

WUBBOLTS, R., LECKIE, R. S., VEENHUIZEN, P. T., SCHWARZMANN, G., MOBIUS, W., HOERNSCHEMEYER, J., SLOT, J. W., GEUZE, H. J. & STOORVOGEL, W. 2003. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *The Journal of biological chemistry,* 278**,** 10963-72.

XIAO, H. & WONG, D. T. 2012. Proteomic analysis of microvesicles in human saliva by gel electrophoresis with liquid chromatography-mass spectrometry. *Analytica chimica acta,* 723**,** 61-7.

YATSUDA, A. P., EYSKER, M., VIEIRA-BRESSAN, M. C. R. & DE VRIES, E. 2002. A family of activation associated secreted protein (ASP) homologues of *Cooperia punctata*. *Research in Veterinary Science,* 73**,** 297-306.

YIGIT, E., BATISTA, P. J., BEI, Y., PANG, K. M., CHEN, C.-C. G., TOLIA, N. H., JOSHUA-TOR, L., MITANI, S., SIMARD, M. J. & MELLO, C. C. 2006. Analysis of the *C. elegans* Argonaute Family Reveals that Distinct Argonautes Act Sequentially during RNAi. *Cell,* 127**,** 747-757.

YOSHINO, T. P., BROWN, M., WU, X. J., JACKSON, C. J., OCADIZ-RUIZ, R., CHALMERS, I. W., KOLB, M., HOKKE, C. H. & HOFFMANN, K. F. 2014. Excreted/secreted Schistosoma mansoni venom allergen-like 9 (SmVAL9) modulates host extracellular matrix remodelling gene expression. *International journal for parasitology,* 44**,** 551-63.

ZAROS, L. G., NEVES, M. R., BENVENUTI, C. L., NAVARRO, A. M., SIDER, L. H., COUTINHO, L. L. & VIEIRA, L. S. 2014. Response of resistant and susceptible Brazilian Somalis crossbreed sheep naturally infected by Haemonchus contortus. *Parasitology research,* 113**,** 1155-61.

ZHAN, B., LIU, Y., BADAMCHIAN, M., WILLIAMSON, A., FENG, J., LOUKAS, A., HAWDON, J. M. & HOTEZ, P. J. 2003. Molecular characterisation of the Ancylostoma-secreted protein family from the adult stage of *Ancylostoma caninum*. *International Journal for Parasitology,* 33**,** 897-907.

ZHAN, B., WANG, Y., LIU, Y., WILLIAMSON, A., LOUKAS, A., HAWDON, J. M., XUE, H. C., XIAO, S. H. & HOTEZ, P. J. 2004. Ac-SAA-1, an immunodominant 16 kDa surface-associated antigen of infective larvae and adults of Ancylostoma caninum. *Int J Parasitol,* 34**,** 1037-45.

ZITVOGEL, L., REGNAULT, A., LOZIER, A., WOLFERS, J., FLAMENT, C., TENZA, D., RICCIARDI-CASTAGNOLI, P., RAPOSO, G. & AMIGORENA, S. 1998. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med,* 4**,** 594-600.

Appendices

Appendix 1. Proteins identified in the exosome-enriched sample. In the table is shown their accession number in the Nembase4 database; their closest homologue after a Blast search; the motifs present in their sequences; and the presence or absence of a signal peptide.

Accession	Blast	Motif	Signal peptide
1. Structural proteins			
ASP00084_11snuclear	*Brugia malayi* actin 2 partial mRNA	Actins	Ø
ASP00084_3snuclear	*Brugia malayi* actin 2 partial mRNA	Actins	Ø
ASP00847_2snuclear	*Globodera rostochiensis* actin 2 mRNA	Actins	Ø
ASP02463_1snuclear	*Dirofilaria immitis* actin mRNA	Actins	Ø
CBP01042_2snuclear	*Caenorhabditis elegans* Protein ACT-4, isoform a	Actins	Ø
CRP02913_1snuclear	*Caenorhabditis elegans* Protein ACT-3 (act-3) mRNA	Actins	Ø
HBP14965_1snuclear	*Microplitis mediator* beta-actin mRNA	Actins	Ø
MHP06728_1snuclear	*Caenorhabditis elegans* Protein ACT-4, isoform b	Actins	Ø

Accession	Blast	Motif	Signal peptide
	(act-4) mRNA		
MJP01140_2snuclear	*Meloidogyne enterolobii* actin mRNA	Actins	Ø
ALP00035_2snuclear	*Loa loa* hypothetical protein (LOAG_12610) mRNA	Actins	Ø
CGP00773_1snuclear	*Strongyloides papillosus* actin variant 2 mRNA, partial cds	Actins	Ø
HGP02959_1snuclear	*Loa loa* beta-actin (LOAG_15273) mRNA	Actins	Ø
OVP09945_1snuclear	*Caenorhabditis elegans* Protein ACT-4, isoform b (act-4) mRNA	Actins	Ø
TMP01181_1snuclear	*Loa loa* hypothetical protein (LOAG_12610) mRNA	Actins	Ø
GRP00004_1	*Caenorhabditis elegans* Protein T24B8.4, isoform a	WH2 actin-binding	Ø
AYP01929_1snuclear	*Ancylostoma caninum* beta-tubulin isotype 2 mRNA	Beta-tubulin	Ø
PVP01960_1snuclear	*Cyathostomum coronatum* beta-tubulin	Beta-tubulin	Ø

Accession	Blast	Motif	Signal peptide
	Cyco-1m mRNA		
HCP02868_1snuclear	*Ostertagia ostertagi* partial mRNA for keratin (ker gene)	Keratin	✓
2. Metabolic Proteins			
SRP00687_2snuclear	*Caenorhabditis elegans* Protein EAT-6 (eat-6) mRNA	E1-E2 ATPases phosphorylation site	Ø
HGP00857_1snuclear	*Globodera rostochiensis* mRNA for peroxiredoxin (tpx gene)	Thioredoxin peroxidase	Ø
3. Nuclear proteins			
CBP03667_2snuclear	*Caenorhabditis elegans* Protein HIS-72, isoform a (his-72) mRNA	Histone	Ø
DVP01908_1snuclear	PREDICTED: *Strongylocentrotus purpuratus* histone H4-like (LOC754479), partial mRNA	Histone	Ø
TMP05225_1snuclear	*Caenorhabditis briggsae* CBR-HIS-71 protein (Cbr-his-71) mRNA	Histone	Ø
BMP07401_1snuclear	*Brugia malayi* elongation factor 1-beta/1-delta	Translation elongation factor	Ø

Accession	Blast	Motif	Signal peptide
	partial mRNA		
4. Activation-associated secreted proteins			
TDP00434_1snuclear	*Ostertagia ostertagi* mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP00472_1snuclear	*Ostertagia ostertagi* mRNA for activation associated secreted protein (al1 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP00533_1snuclear	*Ostertagia ostertagi* mRNA for two-domain activation associated secreted protein ASP4 (asp4)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP02274_1snuclear	*Ostertagia ostertagi* mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP02406_1snuclear	*Ostertagia ostertagi* mRNA for activation associated secreted	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7	✓

Accession	Blast	Motif	Signal peptide
	protein (al1 gene)	family)	
TDP02610_1snuclear	*Heligmosomoides polygyrus* bakeri venom allergen/ancylostoma secreted protein-like 18 gene	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP02887_1	*Heligmosomoides polygyrus* bakeri venom allergen/ancylostoma secreted protein-like 16 gene	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00460_2snuclear	*Ostertagia ostertagi* mRNA for ancylostoma-secreted protein-like protein (aasp2 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00656_1snuclear	*Ostertagia ostertagi* mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00942_2snuclear	*Ostertagia ostertagi* mRNA for ancylostoma-secreted protein-like protein (aasp2 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø

Accession	Blast	Motif	Signal peptide
TDP00942_3snuclear	*Ostertagia ostertagi* mRNA for ancylostoma-secreted protein-like protein (aasp2 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
5. Proteolytic enzymes			
TDP00040_5snuclear	*Teladorsagia circumcincta* secreted cathepsin F (cf1) mRNA	Eukaryotic thiol (cysteine) proteases	✓
TDP02372_1snuclear	*Angiostrongylus cantonensis* putative aspartic protease mRNA	Eukaryotic and viral aspartyl proteases active site	Ø
TDP02397_1snuclear	*Caenorhabditis remanei* hypothetical protein (CRE_19368) mRNA, complete cds / Caenorhabditis elegans Protein ASP-2, isoform b (asp-2) mRNA, complete cds asparatic protease	Eukaryotic and viral aspartyl proteases active site	Ø
TDP02584_1snuclear	*Steinernema carpocapsae* clone Sc-ASP155 aspartic protease precursor, mRNA	Eukaryotic and viral aspartyl proteases active site	Ø
TDP00199_1snuclear	*Ancylostoma ceylanicum* metalloprotease 1	Neutral zinc metallopeptidases	✓

Accession	Blast	Motif	Signal peptide
	precursor (mtp1) mRNA		
TDP03008_1snuclear	*H.contortus* mRNA for zinc metallopeptidase (putative)	Neutral zinc metallopeptidases	Ø
TDP03234_1snuclear	*Haemonchus contortus* putative zinc metallopeptidase (Mep2) mRNA	Neutral zinc metallopeptidases	Ø
6. Excretory / Secretory proteins			
TDP01869_1	*Haemonchus contortus* 15 kDa excretory/secretory protein mRNA	No hit	✓
HCP02856_1snuclear	*Teladorsagia circumcincta* mRNA for excretory secretory protein (ES20 gene)	No hit	Ø
HCP06214_1snuclear	*Teladorsagia circumcincta* mRNA for excretory secretory protein (ES20 gene)	No hit	✓
TDP00176_1snuclear	*Teladorsagia circumcincta* mRNA for excretory secretory protein (ES20 gene)	No hit	Ø
TDP00436_3snuclear	*Ostertagia ostertagi*	No hit	Ø

Accession	Blast	Motif	Signal peptide
	mRNA for putative L3 ES protein		
TDP00589_1snuclear	*Ostertagia ostertagi* mRNA for putative L3 ES protein	No hit	✓
OOP00884_1	*T.colubriformis* 30 kD glycoprotein (ESgp30) mRNA	No hit	Ø
TDP00713_2snuclear	*T.colubriformis* 30 kD glycoprotein (ESgp30) mRNA	No hit	Ø
7. Cell to cell, Cell to matrix interactions			
PEP00051_1	*Caenorhabditis briggsae* CBR-CLC-4 protein (Cbr-clc-4) mRNA	Clc-like (integral membrane component of tight junctions)	Ø
MPP01614_1snuclear	*Loa loa* transmembrane protein (LOAG_00194) mRNA	GOLD domain profile (transmembrane protein)	✓
8. Ribosomal proteins			
ASP13427_1snuclear	*Brugia malayi* 60S ribosomal protein L18 partial mRNA	60S ribosomal protein	Ø
9. Rab GTPases			

Accession	Blast	Motif	Signal peptide
BMP03608_1snuclear	TPA_inf: *Amblyomma variegatum* Rab6 subfamily protein mRNA	Small GTPase Rab1 family profile	Ø
CJP06236_1snuclear	*Caenorhabditis remanei* CRE-RAB-10 protein (Cre-rab-10) mRNA	Small GTPase Rab1 family profile	Ø
PSP01847_2snuclear	*Brugia malayi* Ras-related protein Rab-35 partial mRNA	Small GTPase Rab1 family profile	Ø
PTP00107_1snuclear	*Brugia malayi* Ras-related protein Rab-5B partial mRNA	Small GTPase Rab1 family profile	Ø
XIP05120_1snuclear	*Brugia malayi* Ras-related protein Rab-10 partial mRNA	Small GTPase Rab1 family profile	Ø
10. Other function			
AAP00735_1snuclear	*Loa loa* ADP-ribosylation factor 1 (LOAG_06291) mRNA	ADP-ribosylation factor	Ø
RSP01890_1snuclear	*Loa loa* ADP-ribosylation factor 1 (LOAG_06291) mRNA	ADP-ribosylation factor	Ø
RSP02072_1snuclear	*Loa loa* ADP-ribosylation factor 1 (LOAG_06291) mRNA	ADP-ribosylation factor	Ø

Accession	Blast	Motif	Signal peptide
CSP00154_1snuclear	*Caenorhabditis elegans* Protein TTR-27 (ttr-27) mRNA	Transthyretin-like protein	Ø
HCP06313_1snuclear	*Haemonchus contortus* isolate ISE saposin-like protein 1 mRNA	Saposin B type	Ø
TDP00444_1	*Caenorhabditis remanei* hypothetical protein (CRE_31144) mRNA	Saposin B type	✓
HCP10916_1snuclear	*Brugia malayi* Deoxynucleoside kinase family protein partial mRNA	DB module family protein	Ø
OVP03185_1snuclear	*Brugia malayi* DB module family protein partial mRNA	DB module family protein	✓
OFP01150_1snuclear	*Caenorhabditis remanei* CRE-AIPL-1 protein (Cre-aipl-1) mRNA	Trp-Asp (WD) repeats circular profile	Ø
11. No homology			
ASP00379_1	no hit	No hit	Ø
BMP07902_1	no hit	No hit	Ø
CRP02546_1	no hit	No hit	Ø
DVP00828_1snuclear	no hit	No hit	Ø

Accession	Blast	Motif	Signal peptide
GRP04934_1	no hit	No hit	Ø
HBP08988_1	no hit	No hit	Ø
HCP02887_1	no hit	No hit	Ø
HGP04218_1	no hit	No hit	Ø
HGP11940_1	no hit	No hit	✓
MJP02182_1	no hit	No hit	Ø
OOP00371_1	no hit	No hit	Ø
OOP01914_1	no hit	No hit	Ø
SSP03452_1	no hit	No hit	Ø
TVP00780_1	no hit	No hit	Ø
TDP02702_1	no hit	No hit	✓
TDP02738_1	no hit	No hit	Ø
TDP02964_1	no hit	No hit	Ø

Appendix 2. Proteins identified in the exosome-free ES products. In the table is shown their accession number in the Nembase4 database; their closest homologue after a Blast search; the motifs present in their sequences; and the presence or absence of a signal peptide.

Accession	Blast	Motif	Signal peptide
1. Structural proteins			
AAP00374_1snuclear	Loa loa beta-actin (LOAG_15273) mRNA	Actins	Ø
ASP33249_1snuclear	Dirofilaria immitis actin mRNA	Actins	Ø
CRP04413_1snuclear	Microplitis mediator beta-actin mRNA	Actins	Ø
DVP00123_1snuclear	Caenorhabditis elegans Protein ACT-4, isoform a	Actins	Ø
MHP06936_1snuclear	Microplitis mediator beta-actin mRNA	Actins	Ø
OOP00001_1snuclear	Caenorhabditis brenneri clone 16C1.08 actin (act-1) mRNA	Actins	Ø
TCP00088_1snuclear	Loa loa beta-actin (LOAG_15273) mRNA	Actins	Ø
TMP00013_1snuclear	Trichinella spiralis actin-5C (Tsp_08166) mRNA	Actins	Ø

Accession	Blast	Motif	Signal peptide
XIP00217_1snuclear	Panagrellus redivivus actin mRNA	Actins	Ø
ALP00035_2snuclear	Loa loa hypothetical protein (LOAG_12610) mRNA	Actins	Ø
CGP00773_1snuclear	Strongyloides papillosus actin variant 2 mRNA, partial cds	Actins	Ø
HGP02959_1snuclear	Loa loa beta-actin (LOAG_15273) mRNA	Actins	Ø
OVP09945_1snuclear	Caenorhabditis elegans Protein ACT-4, isoform b (act-4) mRNA	Actins	Ø
TMP01181_1snuclear	Loa loa hypothetical protein (LOAG_12610) mRNA	Actins	Ø
AYP01595_1snuclear	Caenorhabditis elegans Protein LMN-1 (lmn-1) mRNA	Intermediate filaments	Ø
2. Metabolic Proteins			
ACP01204_1snuclear	Angiostrongylus cantonensis enolase mRNA	Enolase	Ø
AYP02969_1snuclear	Angiostrongylus cantonensis enolase mRNA	Enolase	Ø

Accession	Blast	Motif	Signal peptide
PPP03370_1snuclear	Ixodes scapularis hydroxymethylglutaryl-CoA lyase, putative, mRNA	Pyruvate carboxyltransferase	Ø
CBP09379_1snuclear	Caenorhabditis elegans Protein CDC-48.1 (cdc-48.1) mRNA	AAA-protein family	Ø
WBP01030_1snuclear	Caenorhabditis remanei CRE-CDC-48.2 protein (Cre-cdc-48.2) mRNA	AAA-protein family	Ø
CBP03155_1snuclear	Caenorhabditis remanei hypothetical protein (CRE_11766) mRNA	Fructose-bisphosphate aldolase	Ø
TDP00469_1snuclear	Caenorhabditis remanei CRE-ALDO-2 protein (Cre-aldo-2) mRNA	Fructose-bisphosphate aldolase	Ø
WBP01312_1snuclear	Onchocerca volvulus fructose 1,6 bisphosphate aldolase (Fba) mRNA	Fructose-bisphosphate aldolase	Ø
ASP04213_1snuclear	Brugia malayi cyclophilin-type peptidyl-prolyl cis-trans isomerase-15, Bmcyp-5 partial mRNA	Cyclophilin-type peptidyl-prolyl cis-trans isomerase	Ø
BXP05429_1snuclear	Dictyostelium fasciculatum cyclophilin-type peptidylprolyl cis-trans	Cyclophilin-type peptidyl-prolyl cis-trans	Ø

Accession	Blast	Motif	Signal peptide
	isomerase (ppiA) mRNA	isomerase	
SRP01982_1snuclear	Caenorhabditis elegans Protein CYN-5 (cyn-5) mRNA, complete cds	Cyclophilin-type peptidyl-prolyl cis-trans isomerase	Ø
TSP01680_1snuclear	Trichinella spiralis peptidyl-prolyl cis-trans isomerase B (Tsp_03740) mRNA	Cyclophilin-type peptidyl-prolyl cis-trans isomerase	Ø
TVP00084_1snuclear	Trichinella spiralis peptidyl-prolyl cis-trans isomerase B (Tsp_03740) mRNA	Cyclophilin-type peptidyl-prolyl cis-trans isomerase	Ø
SRP05514_2snuclear	Teladorsagia circumcincta glutamate dehydrogenase mRNA	Glutamate dehydrogenases	Ø
SSP03034_1snuclear	Caenorhabditis elegans Protein AHCY-1 (ahcy-1) mRNA	S-adenosyl-L-homocysteine hydrolase	Ø
XIP02435_1snuclear	Caenorhabditis elegans Protein UBQ-1, isoform c (ubq-1) mRNA	Ubiquitin	Ø
XIP05774_1snuclear	Acanthocheilonema viteae ubiquitin (ubi) mRNA, complete cds	Ubiquitin	Ø
HBP01869_1snuclear	Ancylostoma ceylanicum	Thioredoxin	Ø

Accession	Blast	Motif	Signal peptide
	peroxiredoxin-1 gene	peroxidase	
HGP00857_1snuclear	Globodera rostochiensis mRNA for peroxiredoxin (tpx gene)	Thioredoxin peroxidase	Ø
HCP02479_1snuclear	Ovis aries breed Tibetan sheep lysozyme-1 precursor, mRNA	Lysozyme C family	✓
CRP05109_1snuclear	Caenorhabditis remanei CRE-GPI-1 protein (Cre-gpi-1) mRNA	Glucose-6-phosphate isomerase	Ø
HGP09220_1snuclear	Caenorhabditis briggsae C. briggsae CBR-GPI-1 protein (Cbr-gpi-1) mRNA	Glucose-6-phosphate isomerase	Ø
MHP06559_1snuclear	Caenorhabditis elegans Protein GPI-1, isoform a (gpi-1) mRNA	Glucose-6-phosphate isomerase	Ø
CRP06783_1snuclear	Caenorhabditis remanei hypothetical protein (CRE_10816) mRNA	Phosphatidate cytidylyltransferase	Ø
MIP06111_1snuclear	Culex quinquefasciatus dihydrolipoamide dehydrogenase, mRNA / Ascaris suum lipoamide dehydrogenase mRNA, mitochondrial gene encoding mitochondrial	Dihydrolipoamide dehydrogenase	Ø

Accession	Blast	Motif	Signal peptide
	protein, complete cds		
SSP06131_2snuclear	Caenorhabditis briggsae C. briggsae CBR-GPD-1 protein (Cbr-gpd-1) mRNA	Glyceraldehyde-3-phosphate dehydrogenase	Ø
TSP13171_1	Trichinella spiralis putative cysteine synthase A (Tsp_13585) mRNA	Cysteine synthase	Ø
3. Nuclear proteins			
CBP00913_1snuclear	Caenorhabditis remanei CRE-EFT-2 protein (Cre-eft-2) mRNA	Elongation factor	Ø
DVP00256_1snuclear	Caenorhabditis remanei CRE-EFT-2 protein (Cre-eft-2) mRNA	Elongation factor	Ø
4. Stress Proteins			
ACP02548_1snuclear	Caenorhabditis briggsae C. briggsae CBR-HSP-1 protein (Cbr-hsp-1) mRNA	Heat shock hsp70 proteins	Ø
ACP25310_1snuclear	Caenorhabditis briggsae C. briggsae CBR-HSP-1 protein (Cbr-hsp-1) mRNA	Heat shock hsp70 proteins	Ø
BMP12100_1snuclear	O.volvulus heat shock protein 70 mRNA	Heat shock hsp70 proteins	Ø
BXP00182_2snuclear	Bursaphelenchus xylophilus heat shock	Heat shock hsp70	Ø

Accession	Blast	Motif	Signal peptide
	protein (HSP70) mRNA	proteins	
BXP02785_1snuclear	Ditylenchus destructor heat shock protein 70-C mRNA	Heat shock hsp70 proteins	Ø
CJP00280_1snuclear	Bursaphelenchus doui heat shock protein 90 (hsp90-1) mRNA	Heat shock hsp90 proteins	Ø
CRP03786_1snuclear	Caenorhabditis remanei hypothetical protein (CRE_01029) mRNA	Heat shock hsp70 proteins	Ø
HBP00250_1snuclear	Caenorhabditis briggsae C. briggsae CBR-HSP-3 protein (Cbr-hsp-3) mRNA	Heat shock hsp70 proteins	✓
HBP00302_1snuclear	Caenorhabditis briggsae C. briggsae CBR-HSP-1 protein (Cbr-hsp-1) mRNA	Heat shock hsp70 proteins	Ø
HGP00199_1snuclear	Heterodera glycines heat shock protein 70 (HSP70) mRNA	Heat shock hsp70 proteins	Ø
HGP02222_1snuclear	Heterodera glycines heat shock protein 70-C mRNA	Heat shock hsp70 proteins	Ø
MHP00454_1snuclear	Heterodera glycines heat shock protein 70 (HSP70) mRNA	Heat shock hsp70 proteins	Ø
MIP01158_1snuclear	Heterodera glycines heat shock protein 70 (HSP70)	Heat shock hsp70 proteins	Ø

Accession	Blast	Motif	Signal peptide
	mRNA		
PTP01315_1snuclear	Bursaphelenchus mucronatus heat shock protein 90 (hsp90) mRNA	Heat shock hsp90 proteins	Ø
SRP00215_3snuclear	Parastrongyloides trichosuri heat shock 70 protein gene	Heat shock hsp70 proteins	Ø
TSP01927_1snuclear	Trichinella spiralis heat shock protein 90 (Tsp_03752) mRNA	Heat shock hsp90 proteins	Ø
TSP07456_1snuclear	Trichinella spiralis heat shockprotein C (Tsp_03312) mRNA	Heat shock hsp70 proteins	✓
XIP00081_1snuclear	Trichinella nativa heat shock protein 70 (HSP70) mRNA	Heat shock hsp70 proteins	Ø
XIP00142_1snuclear	Toxocara cati heat shock protein 90 (hsp90) mRNA	Heat shock hsp90 proteins	Ø
ASP00457_2snuclear	Ditylenchus destructor 14-3-3 (14-3-3a) mRNA	14-3-3 proteins	Ø
BMP00748_1snuclear	Brugia malayi 14-3-3-like protein 2 partial mRNA	14-3-3 proteins	Ø
CJP02044_1snuclear	Caenorhabditis briggsae C. briggsae CBR-PAR-5 protein (Cbr-par-5) mRNA	14-3-3 proteins	Ø

Accession	Blast	Motif	Signal peptide
HGP05426_1snuclear	Glomus intraradices mRNA for 14-3-3 protein	14-3-3 proteins	Ø
ZPP00029_1snuclear	Bursaphelenchus xylophilus 14-3-3 protein mRNA	14-3-3 proteins	Ø
HCP13213_1snuclear	Caenorhabditis briggsae Hypothetical protein CBG00617 (CBG00617) mRNA	Hypoxia induced protein	Ø
5. Calcium binding proteins			
BMP00928_1snuclear	Caenorhabditis briggsae C. briggsae CBR-CPN-2 protein (Cbr-cpn-2) mRNA	Calponin	Ø
HCP00292_1snuclear	Caenorhabditis elegans Protein ATN-1	Calponin	Ø
6. Activation-associated secreted proteins			
OOP00308_1snuclear	Ostertagia ostertagi mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00435_2snuclear	Ostertagia ostertagi mRNA for ancylostoma-secreted protein-like protein (aasp2 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø

Accession	Blast	Motif	Signal peptide
TDP00435_3snuclear	Ostertagia ostertagi mRNA for ancylostoma-secreted protein-like protein (aasp2 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00447_5snuclear	Ostertagia ostertagi mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00462_1snuclear	Ostertagia ostertagi mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP00462_2snuclear	Ostertagia ostertagi mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP00462_4snuclear	Ostertagia ostertagi mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP00468_3snuclear	Teladorsagia circumcincta mRNA for activation-associated secretory protein	Ancylostoma secreted protein-like (SCP/Tpx-	✓

Accession	Blast	Motif	Signal peptide
	(Asp-1 gene)	1/Ag5/PR-1/Sc7 family)	
TDP00468_5snuclear	Teladorsagia circumcincta mRNA for activation-associated secretory protein (Asp-1 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP00691_1snuclear	Ancylostoma braziliense secreted protein 5 precursor-like mRNA	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP00997_1snuclear	Heligmosomoides polygyrus bakeri venom allergen/ancylostoma secreted protein-like 2 isoform 2 gene	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP01271_2snuclear	Ostertagia ostertagi mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	✓
TDP01414_1snuclear	Heligmosomoides polygyrus bakeri venom allergen/ancylostoma secreted protein-like 5 gene	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø

Accession	Blast	Motif	Signal peptide
TDP02639_1snuclear	Heligmosomoides polygyrus bakeri venom allergen/ancylostoma secreted protein-like 16 gene	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP02996_1snuclear	Heligmosomoides polygyrus bakeri venom allergen/ancylostoma secreted protein-like 14 gene	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00460_2snuclear	Ostertagia ostertagi mRNA for ancylostoma-secreted protein-like protein (aasp2 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00656_1snuclear	Ostertagia ostertagi mRNA for C-type single domain activation associated secreted protein ASP3 precursor (asp3)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00942_2snuclear	Ostertagia ostertagi mRNA for ancylostoma-secreted protein-like protein (aasp2 gene)	Ancylostoma secreted protein-like (SCP/Tpx-1/Ag5/PR-1/Sc7 family)	Ø
TDP00942_3snuclear	Ostertagia ostertagi mRNA for ancylostoma-secreted protein-like protein (aasp2	Ancylostoma secreted protein-like (SCP/Tpx-	Ø

Accession	Blast	Motif	Signal peptide
	gene)	1/Ag5/PR-1/Sc7 family)	
7. Membrane proteins			
MIP03108_1snuclear	PREDICTED: Xiphophorus maculatus canalicular multispecific organic anion transporter 1-like (LOC102218606), mRNA	ABC transporters family	Ø
8. Proteolytic enzymes			
TDP00040_5snuclear	Teladorsagia circumcincta secreted cathepsin F (cf1) mRNA	Eukaryotic thiol (cysteine) proteases	✓
TDP01025_2snuclear	Ostertagia ostertagi mRNA for metalloprotease I (metal I gene)	Neutral zinc metallopeptidases	Ø
TDP01863_1snuclear	Teladorsagia circumcincta mRNA for metalloproteinase (MEP-1 gene)	Neutral zinc metallopeptidases	Ø
TDP01948_1snuclear	Ostertagia ostertagi partial mRNA for metalloprotease	Neutral zinc metallopeptidases	Ø
9. Collagen and collagen-associated proteins			
TDP01447_1	Angiostrongylus cantonensis mRNA for putative COLlagen (col-65	Nematode cuticle collagen	Ø

Accession	Blast	Motif	Signal peptide
	gene)		
10. Globins (heme-binding proteins)			
OOP00190_1snuclear	Ostertagia ostertagi partial mRNA for globin-like ES protein F6	Globin family profile	Ø
11. Excretory / Secretory proteins			
TDP00473_1snuclear	Ostertagia ostertagi partial mRNA for 17 kDa ES antigen protein	No hit	✓
HCP02795_1snuclear	Teladorsagia circumcincta mRNA for excretory secretory protein (ES20 gene)	No hit	Ø
HCP02856_1snuclear	Teladorsagia circumcincta mRNA for excretory secretory protein (ES20 gene)	Casein kinase II phosphorylation site	Ø
HCP06214_1snuclear	Teladorsagia circumcincta mRNA for excretory secretory protein (ES20 gene)	Protein kinase C	✓
TDP00223_1snuclear	Teladorsagia circumcincta mRNA for excretory secretory protein (ES20	Glycosyl hydrolases family 5	Ø

Accession	Blast	Motif	Signal peptide
	gene)		
TDP00176_1snuclear	Teladorsagia circumcincta mRNA for excretory secretory protein (ES20 gene)	No hit	Ø
TDP00723_1snuclear	Ostertagia ostertagi mRNA for putative L3 ES protein	No hit	✓
OOP03925_1snuclear	O.ostertagi mRNA for excretory/secretory antigen	No hit	Ø
TDP00436_3snuclear	Ostertagia ostertagi mRNA for putative L3 ES protein	No hit	Ø
TDP00589_1snuclear	Ostertagia ostertagi mRNA for putative L3 ES protein	No hit	✓
TDP00713_2snuclear	T.colubriformis 30 kD glycoprotein (ESgp30) mRNA	No hit	Ø
12. Other function			
TDP00444_1	Caenorhabditis remanei hypothetical protein (CRE_31144) mRNA	Saposin B type	✓
ACP02775_1smitochondria	Contracaecum osculatum baicalensis voucher CBAI1 cytochrome oxidase subunit II gene	Cytochrome oxidase	Ø

Accession	Blast	Motif	Signal peptide
BMP11202_1snuclear	Synthetic construct clone ATCC 10374254 Rho GDI mRNA	Rho GDP-dissociation inhibitor	Ø
TSP00580_1snuclear	Trichinella spiralis Rab GDP dissociation inhibitor alpha (Tsp_02829) mRNA	Rab GDP-dissociation inhibitor	Ø
HCP12420_1snuclear	Caenorhabditis elegans Protein IDH-1, isoform a	Isocitrate dehydrogenase (NADP(+))	Ø
MCP03763_1snuclear	Loa loa homeobox domain-containing protein (LOAG_10564) mRNA	Homeobox domain protein	Ø
MHP04004_1snuclear	Caenorhabditis remanei CRE-CAH-5 protein (Cre-cah-5) mRNA	Alpha-carbonic anhydrases	Ø
MHP09630_1	Cyathostominae sp. JM-2007a LIM domain protein variant (lim-1) mRNA	LIM domain protein	Ø
OOP00207_1snuclear	Caenorhabditis briggsae C. briggsae CBR-ORA-1 protein (Cbr-ora-1) mRNA	onchocerca related antigen	Ø
TDP00820_1snuclear	Caenorhabditis remanei hypothetical protein (CRE_10152) mRNA	onchocerca related antigen	✓
SRP04421_1snuclear	Brugia malayi Mob1/phocein family	Mob1/phocein family protein	Ø

Accession	Blast	Motif	Signal peptide
	protein partial mRNA		
OFP01150_1snuclear	Caenorhabditis remanei CRE-AIPL-1 protein (Cre-aipl-1) mRNA	Trp-Asp (WD) repeats circular profile	Ø
13. No homology			
ACP07546_1snuclear	no hit	No hit	Ø
ASP13802_1	no hit	No hit	Ø
GPP00397	no hit	No hit	Ø
GRP04934_1	no hit	No hit	Ø
HBP08988_1	no hit	No hit	Ø
HBP18157_1	no hit	No hit	Ø
HGP04218_1	no hit	No hit	Ø
MCP05527_1	no hit	No hit	Ø
MIP03734_1	no hit	No hit	Ø
MJP01213_1	no hit	No hit	Ø
TMP03565_1	no hit	No hit	Ø